약학박사 학위논문

Regulation of Immunoglobulin E-mediated Signal Transduction in Mast Cells by 5, 7, 3′, 4′-Tetrahydroxy-6,8-Diprenylisoflavone from *Cudrania tricuspidata* and Magnolialide from *Laurus nobilis*

꾸지뽕 열매에서 분리한 5,7,3′,4′-Tetrahydroxy-6,8-Diprenylisoflavone 과 월계수 잎에서 분리한 Magnolialide 의 비만세포에서 면역글로블린 E 매개의 신호전달 조절 기전 연구

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Abstract

Regulation of Immunoglobulin E-Mediated Signal Transduction in Mast Cells by 5, 7, 3′, 4′-Tetrahydroxy-6,8-Diprenylisoflavone from *Cudrania tricuspidata* and Magnolialide from *Laurus nobilis*

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Allergic diseases such as atopic dermatitis (AD), asthma, and rhinitis have been characteristically associated with immunoglobulin E (IgE) hyper-production, and mast cells play a crucial role in the development of allergic and inflammatory disorders. Many drugs are based on improvements in existing therapies or on an understanding of the molecular mechanisms involved in allergic diseases. New therapies in development are aimed at inhibiting signals of the allergic inflammatory responses. In this study, we investigated the effects of natural products on IgE-mediated signaling in

mast cells in order to discover new lead compounds for the treatment of allergic disorders.

Cudrania tricuspidata fruit extracts are rich sources of prenylated flavonoids with potential anti-atherosclerotic, hepatoprotective, and anti-inflammatory properties. However, the effect of *Cudrania tricuspidata* fruits extracts and its active compounds on the high affinity IgE receptor (FcεRI)-mediated signaling remains unknown. In this study, the effect of methanol extract from the fruits of *Cudrania tricuspidata* (MFC) and its active compound, 5,7,3′,4′-tetrahydroxy-6,8-diprenylisoflavone (THDPI), on FcεRI-mediated signaling in mast cells were investigated. MFC and THDPI suppressed mast cell degranulation and Ca^{2+} influx. MFC also interfered with IgE-FcεRI interaction and decreased FcεRIβ mRNA expression in mast cells. Furthermore, MFC and THDPI inhibited the phosphorylation of Syk, LAT, and PLCγ, and F-actin redistribution. THDPI also up-regulated Src-like adaptor protein (SLAP) regulating phosphorylated Syk kinase via c-Cbl-dependent ubiquitin-proteasome pathway. These results indicate that MFC and its active compound, THDPI, inhibit mast cell activation through the inhibition of FcεRI-mediated Syk activation, suggesting a therapeutic potential for controlling mast cell activation in inflammatory and/or allergic processes.

This study also investigated the effect of several isolated compounds from *Laurus nobilis* on the inhibition of antigen-induced IgE-mediated mast cell degranulation by measuring the amount of β-hexosaminidase released, and the extent of interleukin (IL)-4 production using a rat basophilic leukemia mast cells (RBL-2H3) in comparison with anti-allergic drug, cromoglycate and ketotifen. Among the seven isolated compounds from *Laurus nobilis*, magnolialide attenuated the β-hexosaminidase release by antigen-induced IgE-activated RBL-3H3 cells, while the other compounds revealed no effects at concentrations tested. Furthermore, magnolialide significantly decreased the IL-4 release in RBL-2H3 cells, and IL-4 mRNA was inhibited. In addition, the inhibition of IL-5-dependent proliferation of early B cells (Y16 cells) by magnolialide was demonstrated. These results suggest that the magnolialide may have beneficial effects for IgE-mediated allergic responses by inhibiting mast cell degranulation, an effector phase of allergic hypersensitivity, and by suppressing IL-4 production and IL-5-dependent early B cell proliferation, key factors in the development and amplification of allergic responses.

Keywords : 5,7,3′,4′-Tetrahydroxy-6,8-diprenylisoflavone, Immunoglobulin E, FcεRI, Mast cell, Magnolialide, SLAP

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Abbreviations

AD	atopic dermatitis
Cbl	casitas b-lineage lymphoma
DNP	dinitrophenyl
FcεRI	high-affinity IgE receptor
HSA	human serum albumin
IgE	immunoglobulin E
IL-4	interleukin-4
IL-5	interleukin-5
LAT	linker of activated T cells
MFC	methanol extracts from the fruits of *Cudrania tricuspidata*
PLCγ	phospholipase Cγ
SLAP	Src-like adaptor protein
Syk	spleen tyrosine kinase
THDPI	5,7,3′,4′-tetrahydroxy-6,8-diprenylisoflavone
Th cell	T helper cell
UPLC	ultra performance liquid chromatography

Chapter 1

Effects of *Cudrania tricuspidata* Fruit Extract and Its Active Compound, 5,7,3′,4′-Tetrahydroxy-6,8-diprenylisoflavone, on the High Affinity IgE Receptor-Mediated Activation of Syk in Mast Cells

1. Introduction

The binding of immunoglobulin E (IgE) to high affinity IgE receptor (FcεRI) on mast cells and basophils is central to the induction of allergic inflammatory responses (Plaut, Pierce et al. 1989). These responses are mediated by cross-linkage of an antigen to IgE bound on FcεRI on the surface of mast cells and basophils. This binding of IgE to FcεRI cross-linked with an antigen causes the aggregation of the FcεRI receptor, which activates the Src family non-receptor tyrosine kinases such as Lyn and Fyn (Ishida, Nishi et al. 2013). Activation of Lyn provokes phosphorylation of spleen tyrosine kinase (Syk) which induces a variety of cellular responses, including mobilization of Ca^{2+},

F-actin polymerization, and activation of the signal transduction pathways, resulting in degranulation of mast cells and cytokine production that ultimately contribute to allergic inflammatory reactions (Gu, Han et al. 2007). It has been demonstrated that Syk has a critical role in IgE-FcεRI-mediated mast cell responses using the overexpression of a mutant form of Syk constructs that abolished the release of inflammatory mediators (Taylor, Karas et al. 1995). Therefore, the inhibition of Syk activation was considered as a therapeutic target in allergic inflammatory diseases (Wong, Grossbard et al. 2004).

The rat basophilic leukemia mast cell line (RBL-2H3), a tumor analog of mast cells, shows phenotypic characteristics of mucosal mast cells and has been widely used as an *in vitro* model for IgE-FcεRI-mediated responses in mast cells (Kim, Lim et al. 2014). After stimulation with an allergen, cells release chemical mediators such as histamine and β-hexosaminidase which are considered to be parameters of mast cell degranulation (Passante and Frankish 2009). Mast cell degranulation results in an acute response, such as the contraction of smooth muscle, vasodilation, and increased vascular permeability (White 1999).

Cudrania tricuspidata (Moraceae) is a deciduous tree widely distributed in

Asia. *Cudrania tricuspidata* has been used as a traditional herbal remedies for anti-tumor, gastritis, and anti-inflammation (Lee, Kim et al. 1996). Recent studies have reported that prenylated flavonoids and xanthones isolated from the root bark of *C. tricuspidata* have anti-atherosclerotic, anti-inflammatory (Park, Park et al. 2006), and hepatoprotective activities (Tian, Kim et al. 2005). The fruits of *C. tricuspidata* have been used as traditional fermented jams, juices, and alcoholic beverages with sugar in Korea. The fruits of *C. tricuspidata* have also been reported to contain prenylated isoflavonoids and benzylated flavonoids with anti-inflammatory and monoamine oxidase inhibitory activities (Han, Hong et al. 2005, Han, Hong et al. 2009). Recent studies have reported that fruits of *C. tricuspidata* show protective effects on oxidative stress-induced neurotoxicity (Jeong, Choi et al. 2010) and antibacterial effects through affecting membrane permeability (Bajpai, Sharma et al. 2013) and inhibit the development of atopic dermatitis (AD)-like skin lesions induced by repeated application of house dust mite (Lee, Ha et al. 2012). In this study, the inhibitory effects of *C. tricuspidata* fruit extract and its active compound, 5,7,3′,4′-tetrahydroxy-6,8-diprenylisoflavone (THDPI), were investigated on mast cell activation through the FcεRI-mediated signaling pathways.

2. Materials and Methods

2.1. Chemicals and reagents

Hyclone™ Minimum Essential Medium with Earle's Balanced Salts (MEM/EBSS) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Fetal bovine serum (FBS) was obtained from Invitrogen (Carlsbad, CA, USA). Monoclonal anti-dinitrophenyl (DNP) IgE mouse antibody, 4-nitrophenyl N-acetyl-β-glucosaminide, DNP-human serum albumin (DNP-HSA), and 2-mercaptoethanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Purified mouse anti-rat FcεRIα-antibody was purchased from BD Biosciences (Franklin Lakes, NJ, USA). *Prime* RT-PCR premix™ was purchased from Genet Bio (Seoul, Korea), and PRO-PREP™ protein extraction solution and easy-BLUE™ total RNA extraction kits were purchased from iNtRON Biotechnology (Kyunggi, Korea). Anti-phospho-Lyn antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-Lyn, anti-phospho-Syk, anti-Syk, anti-phospho-LAT, and anti-LAT antibody, fluorescent Ca^{2+} probe, Fura 2/AM, FITC-avidin, and FITC-conjugated $F(ab')_2$ goat anti-mouse IgG were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA).

2.2. Preparation of methanol extracts from the fruits of *C. tricuspidata* (MFC)

The fruits of *Cudrania tricuspidata* (Moraceae) were collected from the Korea Forest Research Institute, Southern Forest Research Center (Jinju, Korea). A voucher specimen (accession number: KH1-5-090904) was deposited at the Department of Biosystems and Biotechnology, Korea University (Seoul, Korea). Methanol extract from the fruits of *C. tricuspidata* (MFC) was prepared by Professor Dongho Lee at Korea University as follows: The fresh fruits of *C. tricuspidata* (10.7 kg) were sliced and extracted three times with MeOH (10 L × 3) at room temperature. The solvent was evaporated *in vacuo* to obtain a total MeOH extract (630.95 g) (Hiep, Kwon et al. 2014).

2.3. Ultra performance liquid chromatography (UPLC) analysis of MFC

The MFC was analyzed using an Acquity UPLC system (Waters, Millford, MA, USA) with an Acquity UPLC BEH C18 column (1.7 μm, 2.1 × 150 mm i.d.) by Professor Dongho Lee at Korea University. The mobile phase consisted of solvent A (0.05% trifluoroacetic acid in water) and solvent B

(acetonitrile). The starting eluent was 40% B at 0 min. The proportion of B was increased linearly to 100% from 0 to 13 min, held constant at 100% until 14 min, returned to the initial composition (40% B) at 14 min, and then held constant 1 min to re-equilibrate the column. The flow rate was 0.3 mL/min and the sample injection volume was 2 μL. The column and sample managers were maintained at 35 and 15 °C. The UV detection wavelength was monitored at 280 nm.

2.4. Isolation and identification of compounds from MFC

The isolation and identification of ten isoflavones, alpinumisoflavone, 4′-*O*-methylalpinumisoflavone, 5,7,4′-trihydroxy-6,8-diprenylisoflavone, 5,7,3′,4′-tetrahydroxy-6,8-diprenylisoflavone, derrone, erysenegalensein E, isoerysenegalensein E, gancaonin A, warangalone, and osajin from MFC were described in a previous study (Hiep, Kwon et al. 2014).

2.5. Cell cultures

Rat basophilic leukemia (RBL-2H3) cells (ATCC No. CRL-2256) were cultured at 37°C, 5% CO_2 in MEM supplemented with 10% (v/v) fetal bovine

serum, 100 U/mL penicillin, and 100 μg/mL streptomycin.

2.6. Measurement of the release of β-hexosaminidase

Mast cell degranulation was determined by β-hexosaminidase release based on the previously described method.(Lee, Lee et al. 2013) Briefly, RBL-2H3 cells (1 x 10^5 cells/100 μL/well) were cultured in 96-well plates and incubated with monoclonal mouse anti-DNP IgE antibody (anti-DNP IgE) (1 μg/mL) for 24 h at 37°C. The IgE-sensitized RBL-2H3 cells were washed twice with extracellular Hepes buffer (5 mM KCl, 125 mM NaCl, 20 mM Hepes, 1.5 mM $MgCl_2$, 1.5 mM $CaCl_2$). After washing, the cells were treated for 1 h with different concentrations of MFC and isolated compounds in 50 μL of Hepes buffer containing BSA (1%) and glucose (1%) at 37°C and subsequently stimulated with DNP-HSA (500 ng/mL), a multivalent antigen for an additional 1 h. Supernatants (40 μL) were transferred to a 96-well plate and incubated with 50 μL of substrate solution (5 mM of 4-nitrophenyl N-acetyl-β-D-glucosaminide in 50 mM of citric acid buffer, pH 4.5). for 1.5 h at 37°C. The reactions were stopped by the addition of 50 μL of stop solution (0.5 M Na_2CO_3/$NaHCO_3$, pH 10.0) and the absorbance at 405 nm was measured using

a microplate reader (SpectraMax® M5, Molecular Devices, Sunnyvale, CA, USA). The activity value measured from cells treated with IgE/DNP-HSA was considered to represent 100% degranulation.

2.7. Measurement of intracellular Ca^{2+}

RBL-2H3 cells (1 x 10^5 cells/100 μL/well) were cultured in black 96-well plates and incubated with anti-DNP IgE (1 μg/mL) for 24 h at 37°C. Cells were washed with PBS buffer and incubated with Fura 2/AM (20 μM) in 100 μL of Tyrode buffer (5 mM KCl, 130 mM NaCl, 10 mM Hepes, 0.6 mM MgCl, 1 mM $CaCl_2$, 1% BSA, 1% glucose) for 1 h. The cells were washed three times with PBS buffer and treated with different concentrations of MFC and an isolated compound in 100 μL of Tyrode buffer for 15 min and subsequently stimulated with DNP-HSA (500 ng/mL). The fluorescence intensity was recorded for 20 min using a microplate reader (SpectraMax® M5, Molecular Devices, Sunnyvale, CA, USA) at an emission of 512 nm and excitation of 340 and 380 nm. The fluorescence ratio (F_{340}/F_{380}) was used as an indication of a rise in the intracellular concentration of Ca^{2+} (Oh and Lim 2010).

2.8. Measurement of biotinylated-IgE binding to FcεRI

IgE biotinylation (biotinylated-IgE) was carried out according to previously reported methods (Sandomenico, Monti et al. 2011). Briefly, A DNP-IgE (0.5 mg) was dissolved in 500 μL of PBS, and incubated with 50 μL of 10 mM of succinimidyl-6-(biotinamido) hexanoate (Thermo Fisher Scientific, Waltham, MA, USA) dissolved in DMSO. After 1 h of incubation at 4°C, 1 mL of Tris (50 mM, pH 8) was added to deactivate residual reactive groups. The biotinylated-IgE were dialyzed with PBS at 4°C, recovered, and stored frozen until use. For binding experiments, RBL-2H3 cells (1 x 10^6 cells/1 mL/well) were cultured for 24 h in 12-well plates and washed with PBS containing 0.2% bovine serum albumin. The cells were incubated with biotinylated-IgE (2 μg/ml) and MFC and an isolated compound for 1 h at 37°C, detached by gentle scraping, centrifuged at 4,000 rpm for 5 min, suspended in PBS, and stained with 20 μL of FITC-avidin at 4°C for 30 min. As a background control, untreated cells were stained with FITC-avidin alone. The cells were then washed twice with PBS and subjected to analysis of IgE binding to RBL-2H3 cells using a flow cytometer (FACSCalibur, BD Biosciences, San Jose, CA, USA). Values of florescence intensity were obtained from histogram statistic of CellQuest software (BD Biosciences, San Jose, CA, USA).

2.9. Measurement of mRNA expression

RBL-2H3 cells (5 x 10^5 cells/500 μL/well) were incubated for 24 h, treated with MFC and an isolated compound for 3, 6, 18, 24 h (time-dependent), and treated with various concentrations of MFC and an isolated compound for 24 h (dose-dependent). The cells were washed twice with PBS, and total RNA was isolated with easy-BLUE^{TM} (iNtRON Biotechnology, Korea), according to the manufacturer's instruction. cDNA synthesis was performed from 500 ng of total RNA by using ONE-STEP RT-PCR PreMix kit (iNtRoN Biotechnology, Korea). The following primer pairs were synthesized. (Bioneer, Korea).

FcεRIα, 5'-CATTGTGAGTGCCACCATTC-3',
5'-TTCTTCCAGCTACGGCATCT-3';
FcεRIβ, 5'-TTGTCTGCTCCACACTCCAG-3',
5'-AGGCTGCCTCTCACCAGATA-3';
FcεRIγ, 5'-CACCTGTTCTCAGCTGCATT-3',
5'-GGATCAGGGAAGGAAAGAGG-3';
GAPDH, 5'- AGACAGCCGCATCTTCTTGT -3',
5'- CTTGCCGTGGGTAGAGTCAT -3'.

The denaturation, annealing, extension, and cycle conditions were performed as follows: 94°C for 45 s, 62°C for 45 s, 72°C for 45 s, and 26 cycles. The

PCR reaction was performed with a 2720 Thermal Cycler (Applied Biosystems, USA). Aliquots of the PCR reactions were electrophoresed in 1.5% (w/v) agarose gels, and stained with SYBR Safe DNA gel stain (Invitrogen, USA). DNA bands were visualized with LAS 1000plus (FUJIFILM, Japan).

2.10. Measurement of protein expression

RBL-2H3 cells (2 x 10^6 cells/2 mL/well) were cultured in 6-well plates with IgE for 24 h. Cells were then treated with different concentrations of MFC and an isolated compound for 24 h and subsequently stimulated with DNP-HSA (500 ng/ml) for 10 min. Cells were lysed by PRO-PREP™ protein extraction solution (iNtRON Biotechnology, Korea) for 20 min at -20°C. After incubation, lysates were centrifuged at 13,000 rpm for 10 min at 4°C for clarification. Approximately 15 μL (30 μg of total protein) of supernatant was subjected to 10% SDS-PAGE and transferred to PVDF membrane (Immobilon®-P, Millipore, USA). The membrane was incubated with primary antibodies at 4°C overnight. After washing, the membranes were probed with secondary antibodies for 1 h and developed with ECL western blotting detection reagent, WEST-ZOL® plus (iNtRON Biotechnology, Korea). Bands were visualized using an image analyzer, LAS 1000plus (FUJIFILM, Japan).

2.11. Confocal microscopy

RBL-2H3 cells (4 x 10^4 cells) were cultured in cell culture slide (SPL Lifescience, Korea) and sensitized with IgE (2 μg/mL) for 3 h. Before antigen stimulation, RBL-2H3 cells were treated with different concentrations of MFC, an isolated compound, or piceatannol for 1 h, and then stimulated with DNP-HSA (500 ng/mL) for 15 min. Cell fixation was performed with PBS containing 4% formaldehyde for 30 min and permeabilized twice with PBS containing 0.1% Triton X-100 for 5 min. Staining for F-actin was performed with Alexa Fluor® 488 phalloidin (Invitrogen™, USA) for 20 min in the dark. Staining for phosphorylated Syk, SLAP, and c-Cbl were performed with each antibody for 18 h in the dark. DAPI (4',6-diamidino-2-phenylindole) staining was performed to identify all cell nuclei (blue) using a mounting medium containing DAPI (VECTASHIELD® Mounting Medium with DAPI, VECTOR LABORATORIES, USA) prior to analysis by confocal fluorescent microscopy using a LSM 700 (Carl Zeiss, Germany) and ZEN software for the capture of images (Carl Zeiss, Germany).

2.12. Syk kinase activity

Syk kinase activity was measured using ADP-Glo™ Syk Kinase Enzyme System kit (Promega, USA), according to the manufacturer's instruction.

2.13. Proteasome activity

Cell-based proteasome activity was determined using RBL-2H3 cells, as described by Henrik Lovborg and coworkers (Lovborg et al. 2006). Briefly, cells (1.0×10^5 cells/300 μL/well) were cultured in 48-well plates for 24 h with IgE in MEM media supplemented with 10% FBS. Samples were treated for 24 h and the proteolytic activity of the proteasome was evaluated in cell lysates by using a proteasome activity kit (APT 280; Millipore). In brief, 40 μg of cell lysate were incubated for 2 h at 37°C in the provided buffer with fluorophore-linked peptide substrates. We used Suc-LLVY-AMC, Boc-LRR-AMC, and Z-LLE-MCA as the same substrates. Reaction mixtures without cell lysates were used as blanks and AMC or MCA fluorescence was measured at excitation/emission wavelengths of 380/460 and 380/440 nm, respectively.

2.14. SLAP expression constructs

All expression constructs used to express SLAP protein contained murine SLAP cDNA fused to FLAG epitope tag at its NH2 terminus. SLAP cDNA-FLAG inserts were flanked by EcoRI-BamHI sites, and were subcloned into EcoRI-BamHI sites of p3xFLAG-CMV-10, a mammalian expression vector (Sigma-Aldrich, USA). SLAP ΔC expressed a truncated version of SLAP,

including the NH2 terminus, the SH3, and SH2 domains (1-187 amino acids).

2.15. *in vitro* ubiquitination assay

c-Cbl was immunoprecipitated from cells transfected with empty vector or with SLAP WT or SLAP ΔC and used as E3 ligase; Syk was immunoprecipitated from RBL-2H3 cells and used as substrate. The immunoprecipitates were washed separately five times with lysis buffer and then mixed before performing the assay. After an additional wash with ubiquitination buffer (50 mM Tris (pH 7.5), 0.5 mM MgCl2, 0.1 mM ATP, 0.1 mM DTT, 1 mM creatine phosphate), the beads were incubated in 40 μl of the same buffer supplemented with 70% (v/v) rabbit reticulocyte lysates, 10 U of creatine phosphokinase, and 10 μg of Ub for 2 h at 30°C. The samples were washed three times with lysis buffer, eluted with SDS-sample buffer, resolved by SDS-PAGE, and immunoblotted.

2.16. Small interfering RNA (siRNA)

siRNA of SLAP gene was performed using a Control siRNA and SLAP siRNA (sc-37007 and sc-40972, respectively) purchased from Santa Cruz Biotechnology. Transfection with siRNA was performed using

Lipofectamine® 2000 (Invitrogen) according to the manufacturer's instructions.

2.17. Statistical analysis

Data obtained were expressed as the mean ± standard deviation (SD). Statistical significance was determined using GraphPad Prism (GraphPad Software, USA). The differences among the groups were evaluated by one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison method. *P* value of less than 0.05 was considered to be statistically significant. All of the data were obtained from at least three independent experiments.

3. Results

3.1. Inhibitory effects of *Cudrania tricuspidata* on mast cell degranulation

β-Hexosaminidase has been commonly considered as a useful indicator for the evaluation of the activation of mast cells in various allergic inflammation (Pecht and Corcia 1987). As shown in Table 1, methanol extract from the fruits of *C. tricuspidata* (MFC) significantly inhibited the release of β-hexosaminidase in FcεRI-mediated mast cells with an IC_{50} value of 104.9 μg/mL. In the fruit extracts, 47 compounds were isolated and ten major constituents of MFC were identified using UPLC (Figure 1A and 1B). Among ten isolated compounds from MFC, 5,7,3′,4′-tetrahydroxy-6,8-diprenylisoflavone (THDPI) attenuated the release of β-hexosaminidase with an IC_{50} value of 20.4 μM, while the other compounds revealed no significant effects at concentrations up to 40 μM (Figure 2 and Table 2).

Table 1. **Inhibitory effects of parts of *Cudrania tricuspidata* on the release of β-hexosaminidase from mast cells**

Parts of *Cudrania tricuspidata*	**β-hexasaminidase release (IC_{50} μg/mL)**
Fruits	104.9
Leaves	>100[a]
Roots	>100[a]

[a]Cytotoxic at a concentration higher than 100 μg/ml

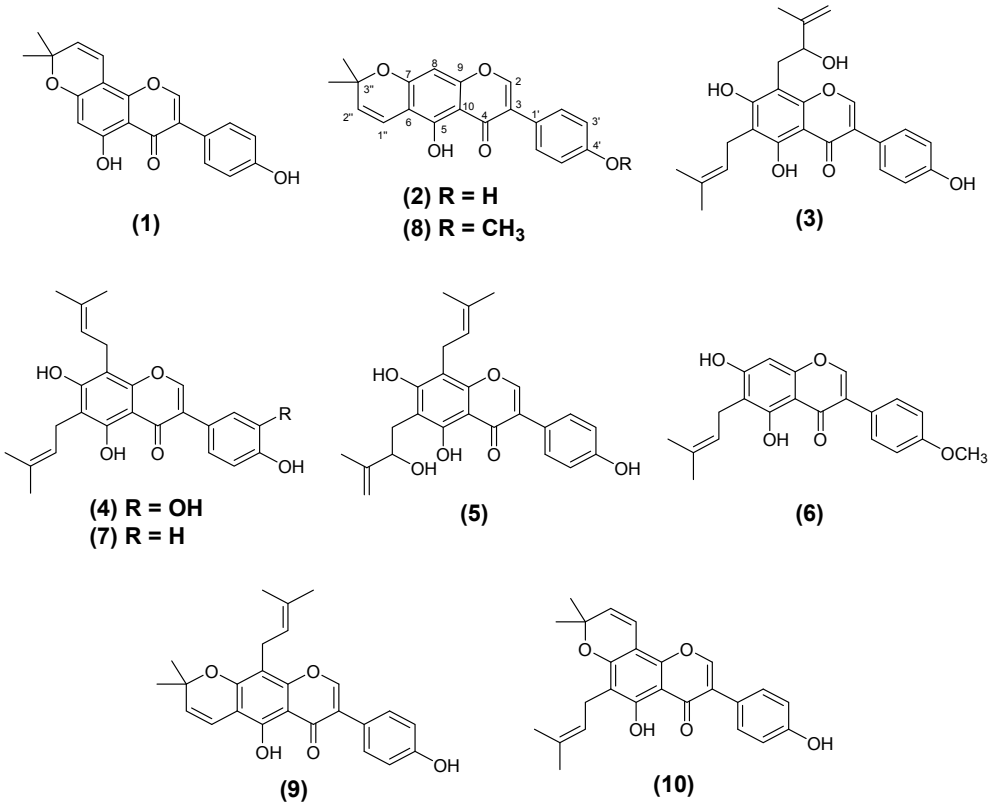


Figure 1A. Chemical structures of principal compounds from methanol extract of the fruits of *Cudrania tricuspidata* (MFC). (A) Chemical structures of principal compounds (B) UPLC chromatogram of MFC. The principal compounds are **(1)** derrone, **(2)** alpinumisoflavone, **(3)** erysenegalensein E, **(4)** 5,7,3′,4′-tetrahydroxy-6,8-diprenylisoflavone, **(5)** isoerysenegalensein E, **(6)** gancaonin A, **(7)** 5,7,4′-trihydroxy-6,8-diprenylisoflavone, **(8)** 4′-*O*-methylalpinumisoflavone, **(9)** warangalone, and **(10)** osajin.

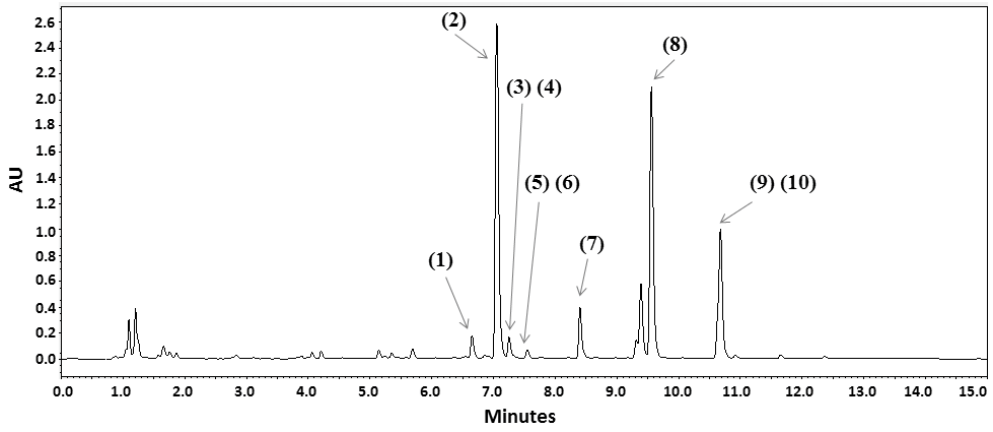


Figure 1B. UPLC chromatogram of *Cudrania tricuspidata* (MFC)

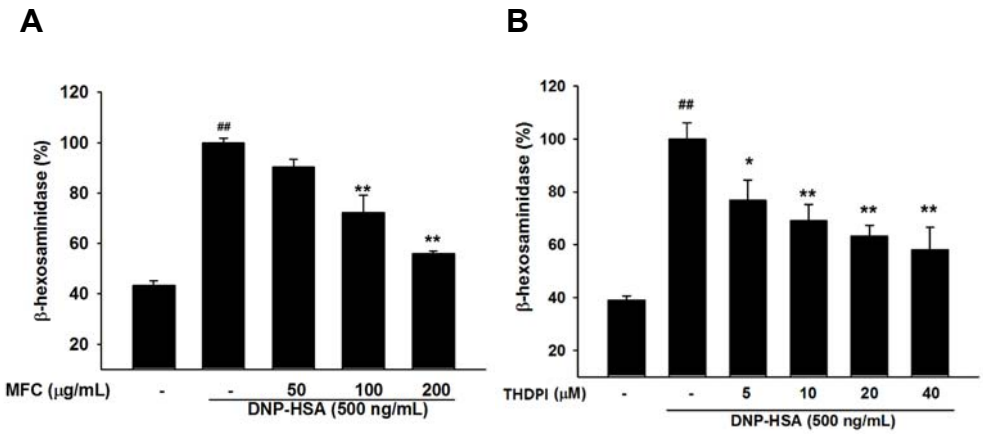


Figure 2. Effect of MFC and THDPI on the mast cell degranulation in RBL-2H3 cells. Anti-DNP IgE-sensitized cells were treated with various concentrations of MFC (A) and THDPI (B) for 1 h followed by an antigen stimulation (DNP-HSA) for an additional 1 h. The release of β-hexosaminidase from cells treated with IgE/DNP-HSA was considered to represent 100% degranulation. Data represent the mean ± SD of three independent experiments, $^{\#\#}p < 0.01$, compared with IgE-sensitized cells without DNP-HSA; $^{**}p < 0.01$, compared with IgE/DNP-HSA-treated cells. THDPI: 5,7,3′,4′-tetrahydroxy-6,8-diprenylisoflavone.

Table 2. **Inhibitory effects of isolated compounds from methanol extract of the fruits of *Cudrania tricuspidata* (MFC) on the release of β-hexosaminidase from mast cells**

Compound	Inhibition of β-hexosaminidase release	
	% at 40 μM	IC_{50} (μM)
(1) Derrone	15.2	>40
(2) Alpinumisoflavone	12.7	>40
(3) Erysenegalensein E	5.6	>40
(4) 5,7,3′,4′-tetrahydroxy-6,8-diprenylisoflavone	57.4	20.4
(5) Isoerysenegalensein E	3.7	>40
(6) Gancaonin A	3.1	>40
(7) 5,7,4′-trihydroxy-6,8-diprenylisoflavone	17.5	>40
(8) 4′-*O*-methylalpinumisoflavone	2.2	>40
(9) Warangalone	8.9	>40
(10) Osajin	4.3	>40
Ketotifen (positive control)	59.4	23.1

IC_{50} values were determined in a semilogarithmic graph with 4 different concentrations of compounds. At least 3 independent experiments were performed. Ketotifen, a mast cell stabilizer, was used as a positive control compound.

3.2. Inhibitory effect of MFC and THDPI on calcium influx

Many anti-allergic drugs are known to inhibit calcium influx into the cytosol, resulting in the suppression of the degranulation of mast cells (Matsubara, Masaki et al. 2004). After the addition of antigen (DNP-HSA) in IgE-sensitized RBL-2H3 cells, the rapid elevation of intracellular calcium influx was observed in the untreated group. However, the treatment of MFC and THDPI decreased the antigen-induced intracellular calcium levels in a dose-dependent manner (Figure 3).

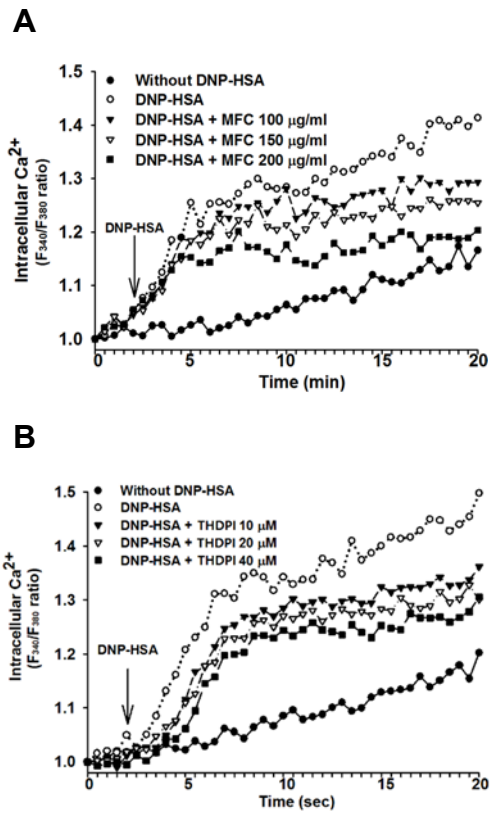


Figure 3. Effect of MFC and THDPI on Ca^{2+} influx in RBL-2H3 cells. Anti-DNP IgE-sensitized cells were incubated with Fura 2/AM for 1 h and thereafter treated with MFC (A) and THDPI (B) for 15 min and subsequently stimulated with an antigen (DNP-HSA, 500 ng/mL). Fluorescence intensity was measured for 20 min. (excitation; 340 and 380 nm, emission; 512 nm). Values were expressed as a ratio of the fluorescence at the two excitation wavelengths (F_{340}/F_{380}). The arrow represents the time at which antigen was added.

3.3. Inhibitory effect of MFC and THDPI on IgE-FcεRI interaction

Binding of IgE to its receptor, FcεRI, on the surface of mast cells is one of the essential steps to initiate mast cell activation and degranulation. Thus, interfering with IgE binding to FcεRI could be another cause to suppress IgE-FcεRI-mediated mast cell activation. When RBL-2H3 cells were treated with MFC at a fixed concentration of IgE, IgE binding levels were decreased to 86.9%, 79.3%, and 64.7% when treated with MFC at 100, 150, and 200 μg/mL, respectively, compared with control groups treated with IgE alone (Figure 4A). However, the treatment of THDPI showed no significant effects at concentrations up to 40 μM in IgE-FcεRI interaction (Figure 4B).

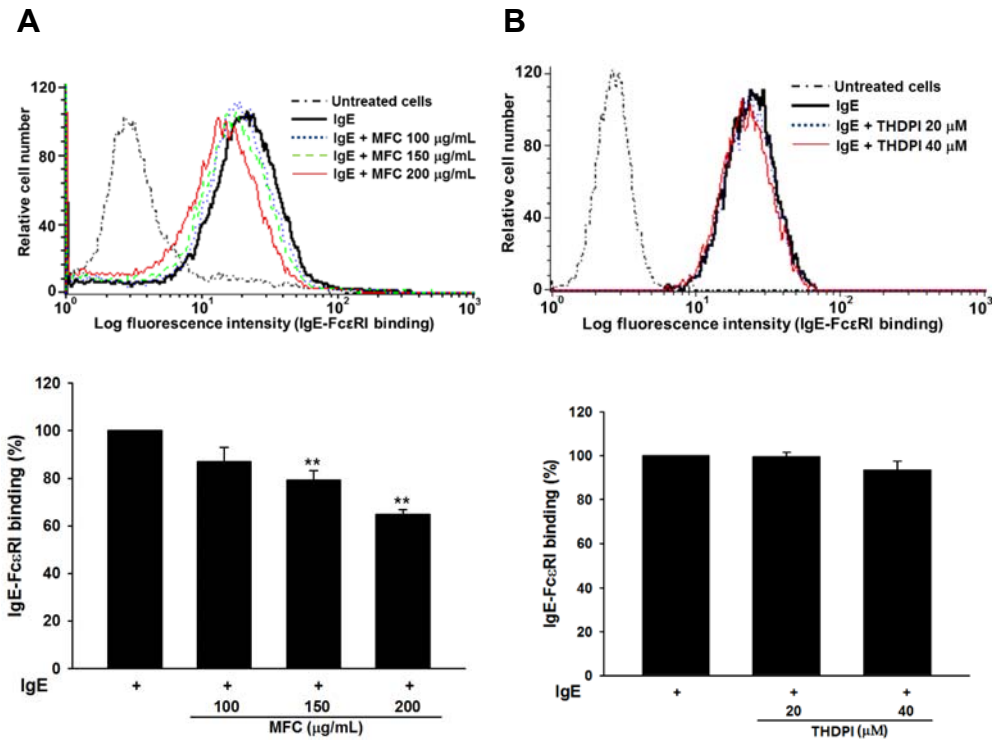


Figure 4. Effect of MFC and THDPI on IgE-FcεRI binding. RBL-2H3 cells were treated with biotinylated-IgE (2 μg/mL) and various concentrations of MFC (A) and THDPI (B) for 1 h. The cells were then stained with FITC-avidin for 30 min. IgE bound to FcεRI on RBL-2H3 cells was analyzed employing flow cytometry. Quantitative comparison of IgE-FcεRI binding between IgE-treated cells and sample-treated cells. Values of florescence intensity were obtained from histogram statistic of CellQuest software. Data represent the mean ± SD of three independent experiments, $^{**}p < 0.01$, compared with the IgE-treated cells.

3.4. Inhibitory effect of MFC and THDPI on FcεRI mRNA expression

FcεRI are multimeric immune receptors consisting of one α-subunit, one β-subunit, and two γ-subunits. The α-subunit binds to the Fc portion of IgE, and β- and γ-subunits are thought to account for the cell-activation properties of FcεRI (Kraft and Kinet 2007). To examine whether MFC and THDPI have an effect on FcεRI expression in mast cells, RBL-2H3 cells were treated with MFC and THDPI for 24 h. MFC inhibited the only FcεRI β-subunit mRNA expression significantly, however mRNA expression of the α- and γ-subunits were not affected (Figure 5A and 5C). In the group treated with THDPI, however, no inhibitory effect on FcεRI subunits was observed (Figure 5B and 5D).

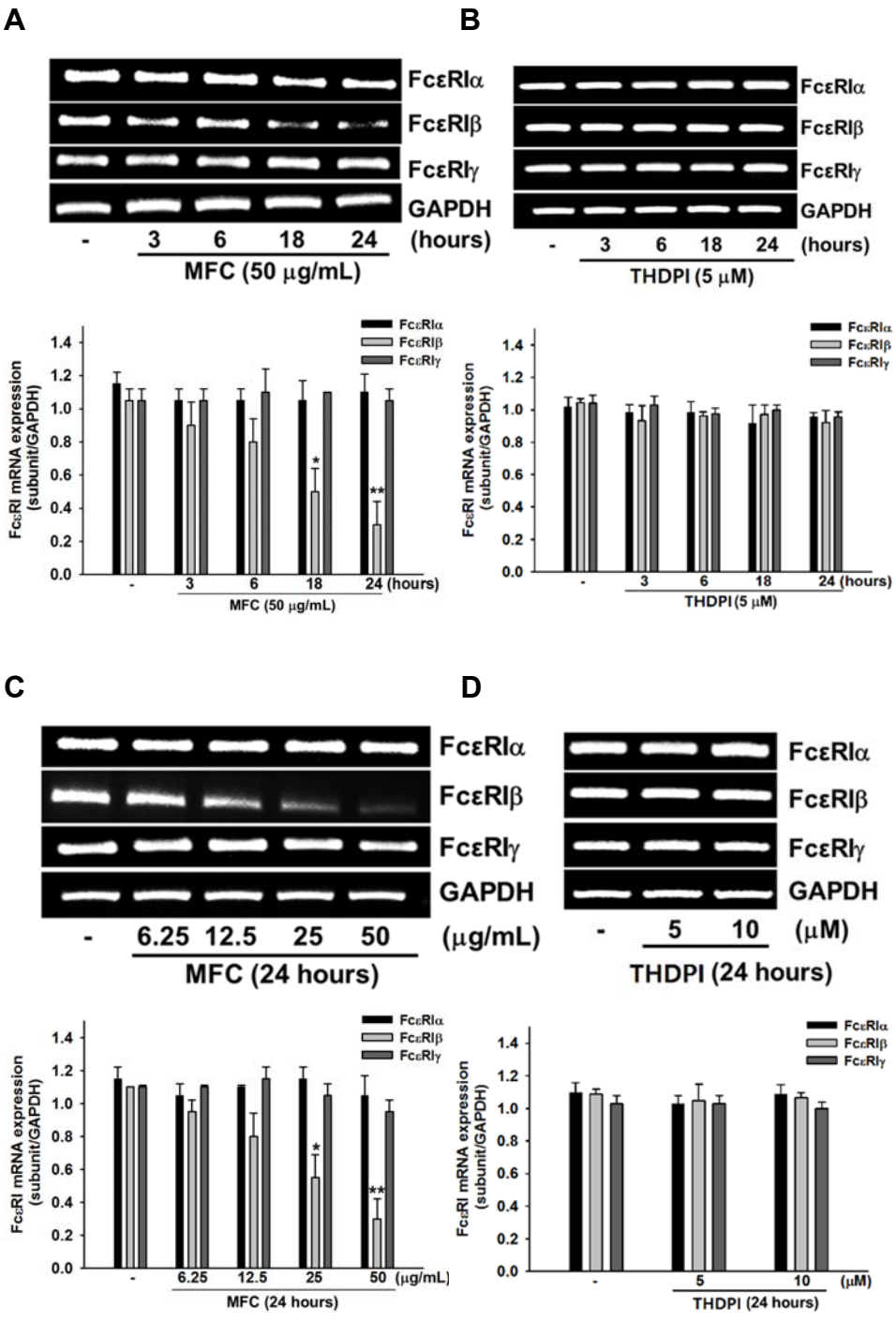
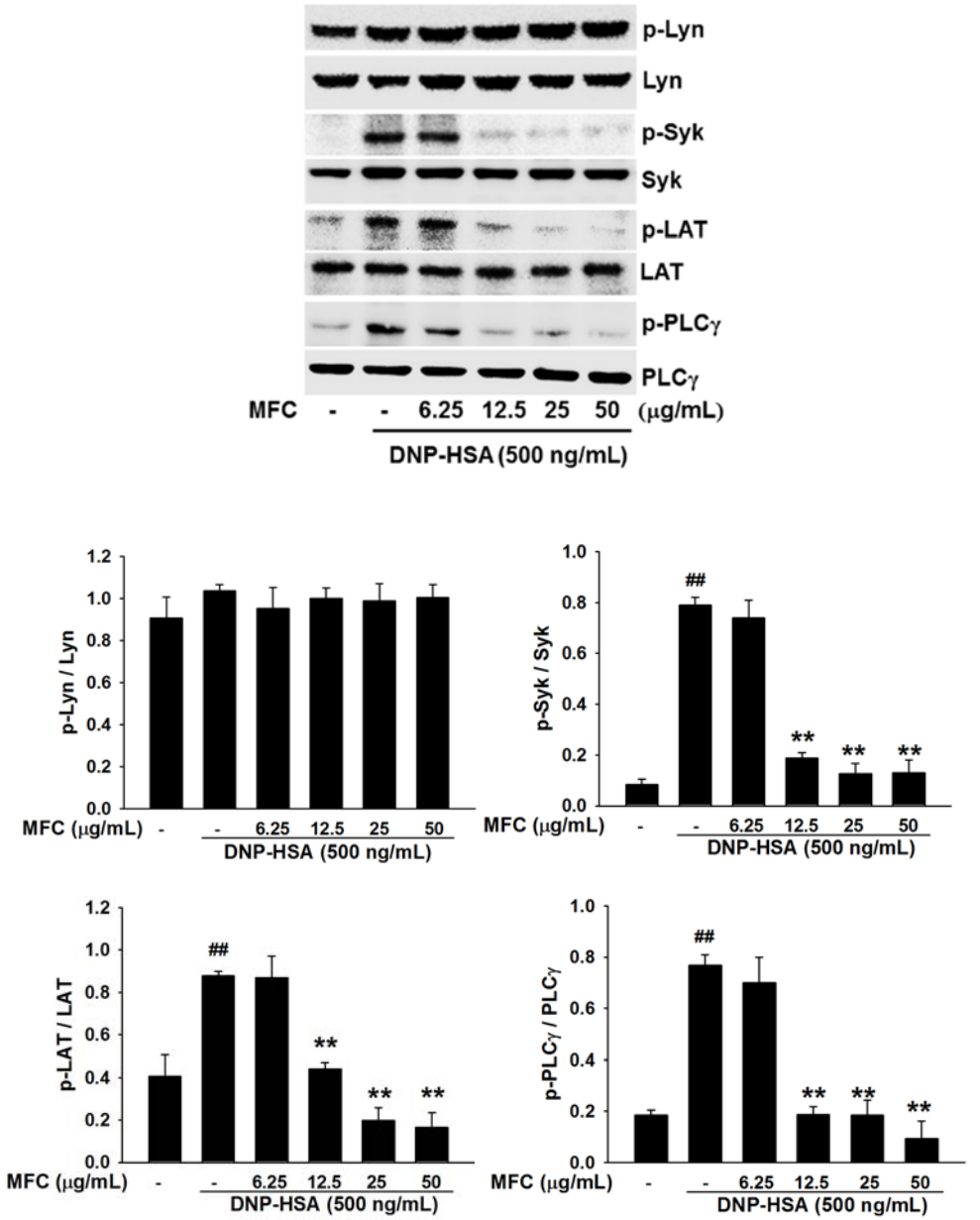

A
B
FcεRIα
FcεRIβ
FcεRIγ
GAPDH
- 3 6 18 24 (hours)
MFC (50 μg/mL)
THDPI (5 μM)
FcεRI mRNA expression (subunit/GAPDH)
C
D
- 6.25 12.5 25 50 (μg/mL)
MFC (24 hours)
- 5 10 (μM)
THDPI (24 hours)

Figure 5. Effect of MFC and THDPI on FcεRI mRNA expression in RBL-2H3 cells. RBL-2H3 cells were treated with MFC and THDPI for different time intervals (0, 3, 6, 18, and 24 h) (A and B). RBL-2H3 cells were treated with various concentrations of MFC and THDPI for 24 h (C and D). The mRNA expression levels of FcεRI-subunit genes, α, β, and γ, were determined by RT-PCR analysis. Quantitative representation of the expression of each subunit of FcεRI and GAPDH mRNA using Image J software. Data represent the mean ± SD of three independent experiments, $^{*}p < 0.05$, $^{**}p < 0.01$, compared with the non-MFC-treated cells.

3.5. Inhibitory effect of MFC and THDPI on FcεRI signaling

Cross-linking of the FcεRI on mast cells activates non-receptor type protein tyrosine kinases such as Lyn and Syk (Itoh, Ninomiya et al. 2009). LAT is known to be phosphorylated by Syk and is important for activation of PLCγ, which is essential for increasing the intracellular calcium level in the calcium-dependent pathway (Huang, Yamaki et al. 2008). As demonstrated in Figure 6A and 6B, both MFC and THDPI showed no effect on the phosphorylation of Lyn, however, they suppressed the phosphorylation of Syk as well as its downstream effectors, LAT and PLCγ in IgE-sensitized RBL-2H3 cells stimulated by antigens. A previous study has reported that piceatannol, a protein kinase inhibitor, reduced the antigen-induced secretion in IgE-sensitized mast cells through the inhibition of phosphorylation of Syk without the inhibition of phosphorylation of Lyn (Oliver, Burg et al. 1994).

A


p-Lyn
Lyn
p-Syk
Syk
p-LAT
LAT
p-PLCγ
PLCγ
MFC - - 6.25 12.5 25 50 (μg/mL)
DNP-HSA (500 ng/mL)
p-Lyn / Lyn
p-Syk / Syk
p-LAT / LAT
p-PLCγ / PLCγ
##
**
MFC (μg/mL) - - 6.25 12.5 25 50
DNP-HSA (500 ng/mL)

B

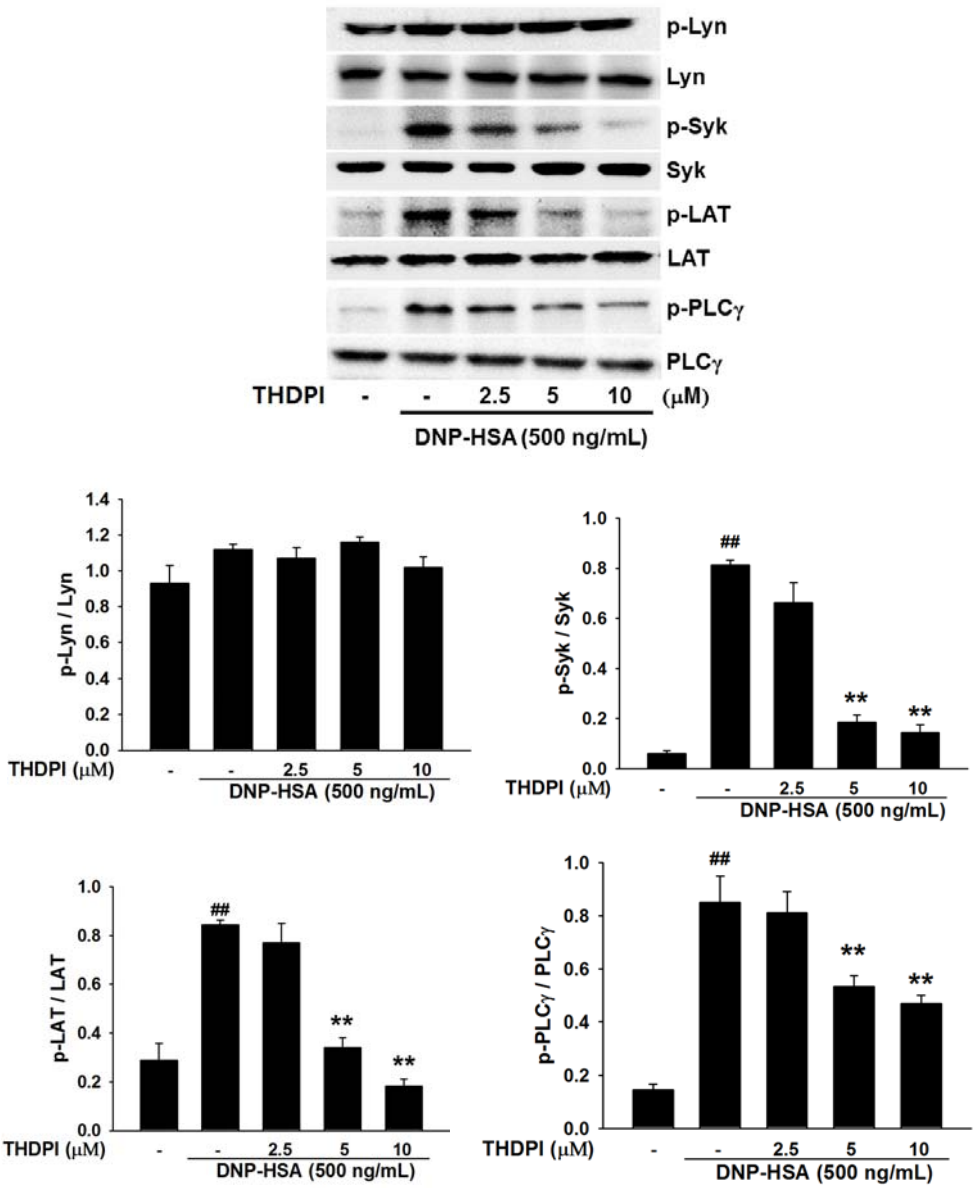

p-Lyn
Lyn
p-Syk
Syk
p-LAT
LAT
p-PLCγ
PLCγ
THDPI - - 2.5 5 10 (μM)
DNP-HSA (500 ng/mL)
p-Lyn / Lyn
p-Syk / Syk
p-LAT / LAT
p-PLCγ / PLCγ
##
**
THDPI (μM)
DNP-HSA (500 ng/mL)

Figure 6. Effect of MFC and THDPI on the FcεRI-mediated phosphorylation of Lyn, Syk, LAT, and PLCγ in RBL-2H3 cells. Anti-DNP IgE-sensitized RBL-2H3 cells were cultured with various concentrations of MFC (A) and THDPI (B) for 24 h followed by antigen stimulation for 10 min. Then, each cell lysate was subjected to immunoblot analysis. The p-Lyn, p-Syk, p-LAT, and p-PLCγ represent the phosphorylated Lyn, Syk, LAT, and PLCγ, respectively. Data represent the mean ± SD of three independent experiments, $^{\#\#}p < 0.01$, compared with IgE-sensitized cells without DNP-HSA; $^{**}p < 0.01$, compared with IgE/DNP-HSA-treated cells.

3.6. Effect of MFC and THDPI on F-actin redistribution

The FcεRI-mediated signaling in mast cells leads not only to degranulation and cytokine production, but also to actin assembly and various morphological changes in the cells. In Figure 7, these morphological changes were observed in RBL-2H3 cells stained with fluorescent phalloidin to visualized F-actin. In unstimulated cells, F-actin (green) was at steady state and concentrated at the cell membrane. When stimulated with antigen (DNP-HSA), F-actin was redistributed and cells were spread (Figure 7). However, this redistribution of F-actin was markedly inhibited in MFC-treated cells (Figure 8C and 8D) and in THDPI-treated cells (Figure 8E and 8F). These inhibitory effects on F-actin redistribution were compared with a Syk inhibitor, piceatannol (Figure 8G), which has been reported to inhibit actin assembly, membrane ruffling, spreading, and degranulation in mast cells (Cox, Chang et al. 1996).

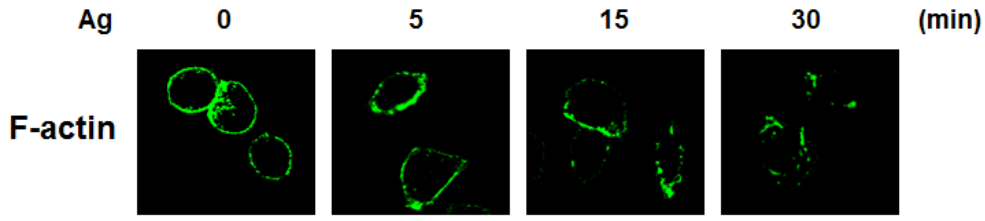


Figure 7. Effect of antigen stimulation on F-actin assembly in RBL-2H3 cells. IgE-sensitized RBL-2H3 cells were stimulated with Ag (DNP-HSA, 500 ng/ml) for 5, 15, or 30 min. Cells were fixed and then double stained with fluorescent phalloidin for F-actin (green).

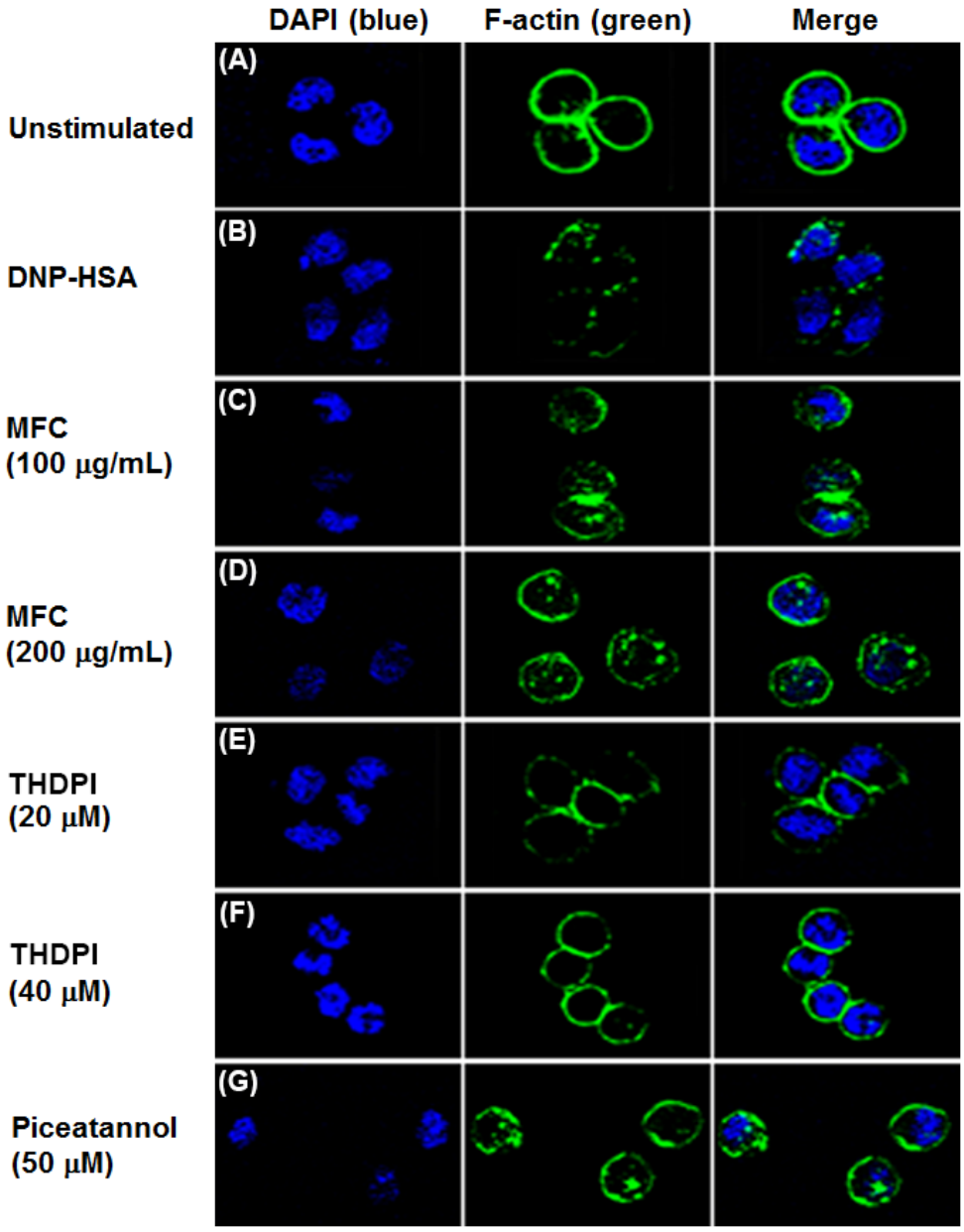

DAPI (blue)
F-actin (green)
Merge
(A)
Unstimulated
(B)
DNP-HSA
(C)
MFC
(100 μg/mL)
(D)
MFC
(200 μg/mL)
(E)
THDPI
(20 μM)
(F)
THDPI
(40 μM)
(G)
Piceatannol
(50 μM)

Figure 8. Effect of MFC and THDPI on F-actin assembly in RBL-2H3 cells. IgE-sensitized RBL-2H3 cells were treated with MFC, THDPI, and piceatannol for 1h followed by antigen stimulation (DNP-HSA, 500 ng/ml) for 15 min. Cells were fixed and then double stained with fluorescent phalloidin for F-actin (green) and DAPI for cell nuclei (blue). Panels show representative images from (A) unstimulated cells, (B) cells stimulated with antigen, (C and D) MFC-treated cells stimulated with antigen, (E and F) THDPI-treated cells stimulated with antigen, (G) piceatannol-treated cells stimulated with antigen. Original magnification, ×400.

3.7. Effect of THDPI on Syk kinase and proteasome activity

Syk kinase is one of non-receptor protein tyrosine kinase and essential elements in several cascades connecting immune receptors to intracellular responses. Thus, several studies have investigated a negative feedback loop regulating Syk activity. Herein, we also investigated the mechanism actions of THDPI on Syk activity. Syk did not show any significant inhibitory effects on *in vitro* Syk kinase activity (Figure 9). However, MG132, a proteasome inhibitor, induced accumulation of phosphorylated Syk upon Ag-stimulation (Figure 10A). MG132 also reversed the inhibitory effects of THDPI on phosphorylation and ubiquitination of Syk (Figure 10B, 10C). We also conducted an experiment to evaluate whether THDPI affect proteasome activity or not. As shown in Figure 11, THDPI did not show any effects on proteasome activity.

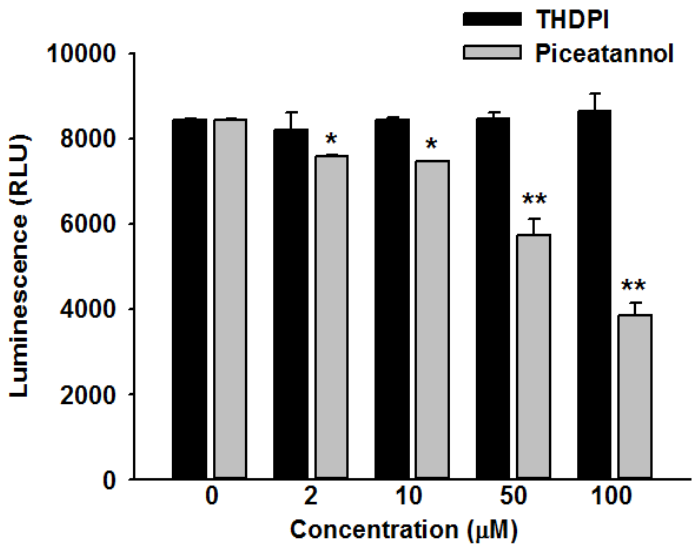


Figure 9. Effect of THDPI on Syk kinase activity. Syk kinase reactions were incubated with the indicated concentrations of THDPI or piceatannol, which is a known inhibitor of Syk kinase, for 60 min at room temperature. The reactions were performed at 10 μM ATP and substrate (poly-glu4-tyr1, 1 mg/mL). Data represent the mean ± SD of three independent experiments, $^{*}p < 0.05$, $^{**}p < 0.01$, compared with the non-treated cells.

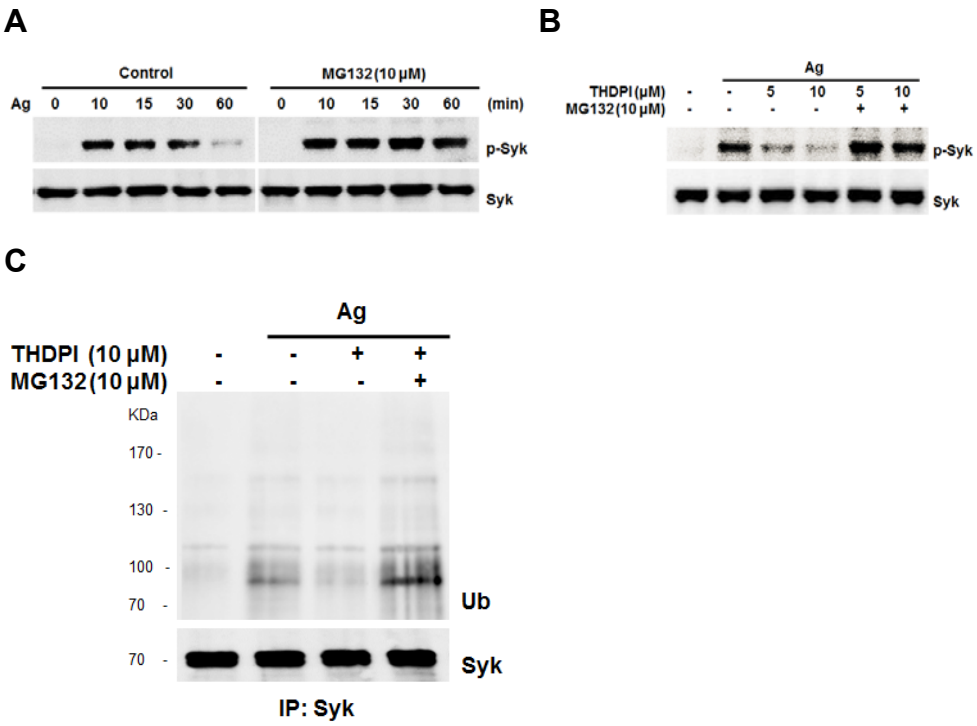


Figure 10. The Proteasome inhibitor, MG132, induces accumulation of phosphorylated Syk. RBL-2H3 cells were treated with MG132 (10 μM) for 5 h, and then stimulated with antigen (DNP-HSA, 500 ng/mL) for the indicated time periods (A). RBL-2H3 cells were treated with or without the indicated concentrations of THDPI following treatment with MG132 for 5 h or not. Then, each cell lysate was subjected to immunoblot analysis (B). RBL-2H3 cells were treated with or without THDPI (10 μM) following treatment with MG132 or not. The total cell lysates were immunoprecipitated with Syk antibody and immnoblotted with Ub antibody (C).

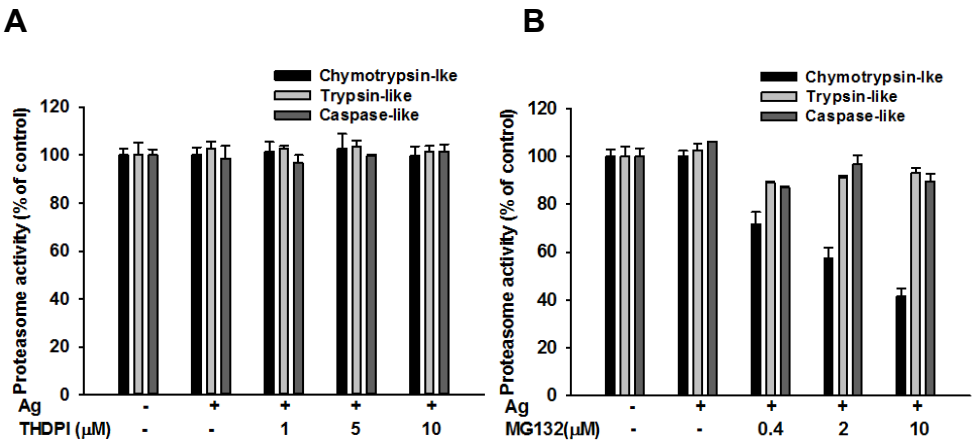


Figure 11. Effects of THDPI on proteasome activities. Fluorogenic peptides used as substrates were Suc-LLVY-AMC (40 μM), Boc-LRR-AMC (40 μM), and Z-LLE-MCA (80 μM) for chymotrypsin-like, trypsin-like, and caspase-like proteases activities, respectively. Proteasome activity by THDPI (A) or MG132 (B) is given as a percentage of that of non-treated cells, and data represent the mean ± SD of three independent experiments.

3.8. Effect of SLAP expression on Syk kinase

Src-like adaptor protein (SLAP) is reported to negatively regulate T cell function by down-regulating expression of TCR and TCR signaling by associating with Zap70 and c-Cbl. SLAP possesses Src homology (SH)2 and SH3 domains that are homologous to those in Src family kinases including Syk (Hiragun, Peng et al. 2006). We investigated the effect of THDPI on SLAP expression in RBL-2H3 cells. THDPI up-regulated SLAP mRNA expression and protein levels (Figure 12). Using SLAP expression constructs including a truncated version of SLAP (ΔC), we found that SLAP overexpression inhibited the Ag-stimulated phosphorylation of Syk and it was mainly due to the action of the ubiquitin ligase c-Cbl binding to c-terminus of SLAP (Figure 13B). Suppression of the induction of SLAP by siRNA also reversed the inhibitory effects of THDPI on phosphorylated Syk (Figure 13C). We also compared c-Cbl ligase activity in SLAP WT and SLAP ΔC by immunoprecipitating the c-Cbl from total extracts and performing an in vitro ubiquitination assay. We used rabbit reticulocyte lysates (RRL) as source of E1 and E2 enzyme and Syk immunoprecipitated from RBL-2H3 cells as substrate. An increase of Syk ubiquitination was observed when c-Cbl ligase was immnoprecipitated from cells transfected with SLAP WT (Figure 14A).

SLAP/c-Cbl complex was also observed from SLAP WT-overexpressed cell lysates compared to SLAP ΔC (Figure 14B). The colocalization of SLAP and c-Cbl was also investigated using confocal microscopy (Figure 15).

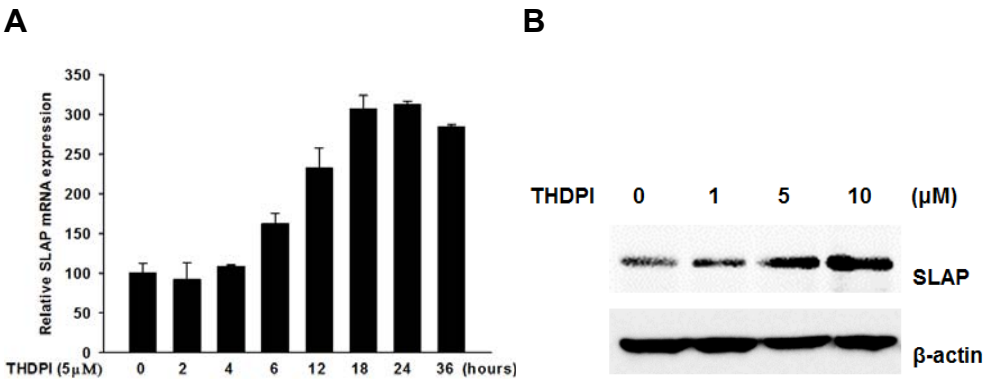


Figure 12. Effect of THDPI on SLAP expression. RBL-2H3 cells were treated with THDPI for the indicated time periods and concentrations. The levels of SLAP mRNA (A) and SLAP protein (B) expressions were analyzed using real-time PCR and immunoblotting, respectively.

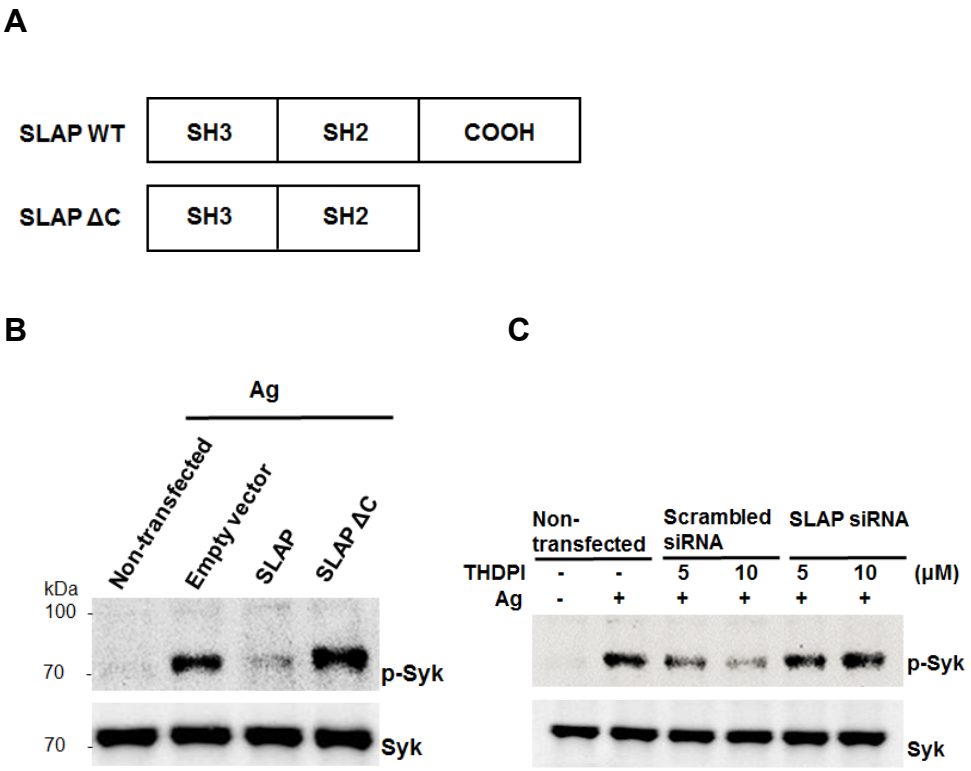


Figure 13. THDPI attenuates Ag-stimulated phosphorylated Syk as does the overexpression of SLAP. SLAP expression construct: SLAP wild type (WT) and deletion of C terminus (ΔC) (A). RBL-2H3 cells were transiently transfected with empty vector, SLAP WT, or SLAP ΔC and then incubated with IgE for 24 h. The cell were stimulated or not with Ag for 10 min (B). RBL-2H3 cells, transfected with scrambled siRNA and SLAP siRNA, were incubated with IgE, with the indicated concentrations of THDPI for 24 h (C). Then, each cell lysate was subjected to immunoblot analysis

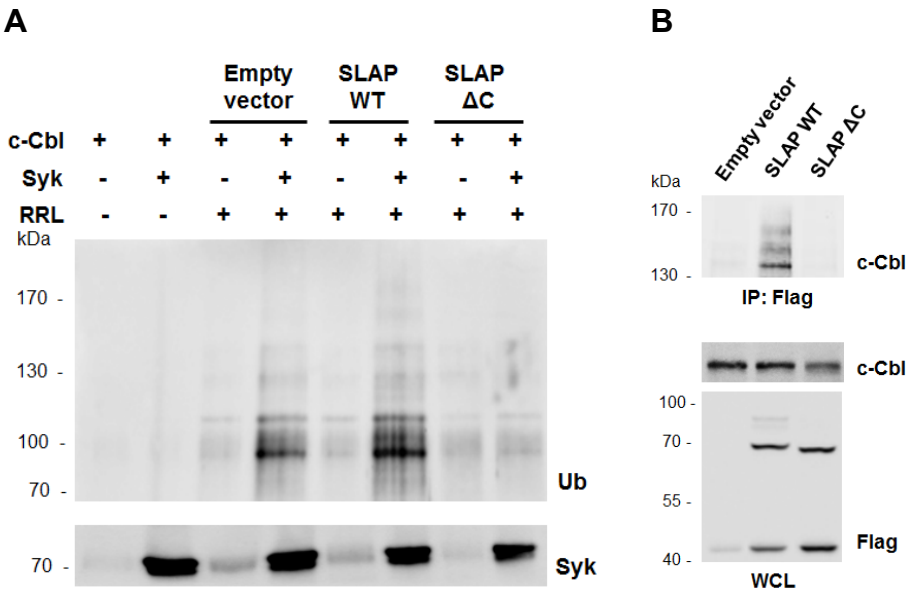


Figure 14. c-Cbl ligase activity and SLAP/c-Cbl complex formation upon SLAP overexpression. c-Cbl immuneprecipitated from RBL-2H3 cells transfected with empty vector, SLAP WT, or SLAP ΔC was used in an *in vitro* ubiquitination assay. Syk immunoprecipitated from RBL-2H3 cells was used as substrate. Protein A-Sepharose beads conjugated with anti-c-Cbl and anti-Syk antibodies were used in the absence of cell extracts as negative control. After the reaction proteins were immnoblotted with the indicated antibodies (A). RBL-2H3 cells transfected with empty vector, SLAP WT, or SLAP ΔC were immunoprecipitated with anti-FLAG antibody, immnoblotted with the indicated antibodies (B). RRL indicates E1, E2 enzyme sources.

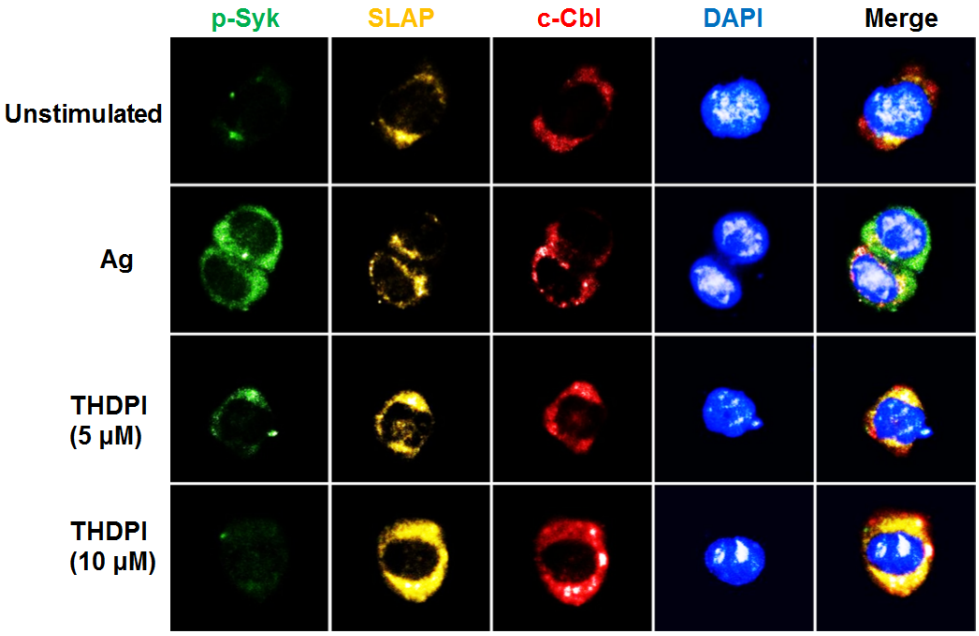


Figure 15. Expression and localization of SLAP and c-Cbl. RBL-2H3 cells were treated with THDPI for 24 h followed by antigen stimulation for 15 min. Cells were fixed and then stained with p-Syk (green), SLAP (yellow), c-Cbl (red), and DAPI for cell nuclei (blue).

4. Discussion

Mast cells are crucial factors in the regulation of inflammatory and allergic responses due to their ability to produce pro-inflammatory mediators. The major mechanism for the activation of mast cells is the interaction of immunoglobulin E (IgE) with its high affinity receptor (FcεRI), on the cell surface, which initiates FcεRI signaling through Lyn and Syk to regulate degranulation (Vial, Oliver et al. 2003). In this study, we investigated the inhibitory effects of methanol extract from the fruits of *C. tricuspidata* (MFC) on FcεRI-mediated mast cell activation and identified active compounds responsible for the inhibitory action. Among ten isolated compounds in this study, the major difference between the active compound, 5,7,3′,4′-tetrahydroxy-6,8-diprenylisoflavone (THDPI), and others is the hydroxyl groups at the 3′- and 4′-positions. In comparison with compound (**7**), the absence of hydroxyl group at 3′-position weaken the inhibitory activity on degranulation. However, it is necessary to further explore this to determine exact structure-activity relationships. To investigate the mechanism underlying the inhibitory effects of MFC and THDPI on mast cell degranulation, free cytoplasmic calcium levels were examined. The movement of calcium across the membrane into the cytosol, either from an

intracellular store such as the endoplasmic reticulum or from the external environment, plays a crucial role in IgE-mediated mast cell activation and degranulation (Pecht and Corcia 1987). MFC and THDPI suppressed intracellular calcium ion elevation, which elicit its inhibitory effects on mast cell degranulation at least in part by the inhibition of calcium influx into the cytosol.

It has been reported that omalizumab, a recombinant DNA-derived humanized IgG1k monoclonal antibody, interferes with the IgE-FcεRI binding and inhibit mast cell degranulation (MacGlashan, Bochner et al. 1997), and it is currently used for the control of allergic asthma and atopic dermatitis (Velling, Skowasch et al. 2011). In FcεRI knockout mice, binding of IgE to mast cells was completely blocked causing no induction of degranulation following allergic reactions (Dombrowicz, Flamand et al. 1993). Also, modulation for FcεRI expression could be considered as a therapeutic target for preventing FcεRI-mediated allergic inflammatory diseases. Recently, epigallocatechin gallate (EGCG) has been reported as a possible inhibitor for FcεRI expression by inhibiting mRNA expression of γ-subunit in mast cells (Tamura, Yoshihira et al. 2010). Therefore, inhibition of IgE-FcεRI binding and FcεRI expression could be crucial steps to block signaling cascades initiated by IgE. MFC interfered with IgE-FcεRI binding and down-regulated

FcεRIβ expression in a dose-dependent manner, however, THDPI did not show any significant effects on IgE-FcεRI binding and FcεRI expression. It is possible that other minor compounds in MFC may be responsible for the inhibitory effects on the expression of IgE receptors and the affinity of IgE to the receptors. It is necessary to identify more minor compounds from MFC to explain these mechanistic differences between MFC and THDPI.

Lyn can be constitutively associated with FcεRI and is responsible for the phosphorylation of β- and γ-subunits. These phosphorylation events result in the association of Syk with phosphorylated γ-subunit, thereby promoting the activation of Syk (Knol 2006). It has been reported that Syk-deficient mast cells completely abrogated the degranulation, calcium elevation, and activation of the ERK and JNK (Itoh, Ninomiya et al. 2009). In LAT-deficient bone marrow-derived mast cells (BMMCs), tyrosine phosphorylation of FcεRI and Syk was intact following FcεRI engagement, however, activation of PLCγ and calcium mobilization were dramatically reduced (Saitoh, Arudchandran et al. 2000). MFC and THDPI showed significant inhibitory effects on phosphorylated Syk, Lat, and PLCγ, but not on phosphorylated Lyn. These data suggested that the inhibitory mechanism of MFC and THDPI may be capable of regulating Syk activation in mast cells.

The morphological changes involve the redistribution of F-actin and are in

parallel with degranulation of mast cells (Oliver, Sahara et al. 1992). Several studies have demonstrated that these morphological changes are down-stream of Syk and are Ca^{2+} dependent (Sahara, Siraganian et al. 1990, Siraganian, de Castro et al. 2010). It has been also reported that activation of FcεRI induces no or minimal morphological change in $Syk^{-/-}$ cells, but this is reconstituted by stable expression of Syk in these cells (Siraganian, de Castro et al. 2010). In addition, IgE-mediated redistribution of F-actin is observed only in $Syk^{-/-}$ cells transfected with wild-type Syk (de Castro 2011). MFC and THDPI inhibited redistribution of F-actin induced by antigen stimulation. These inhibitory effects on F-actin redistribution supports that MFC and THDPI may regulate Syk activation which modulate IgE-mediated mast cell activation.

Ubiquitination and the consequent degradation of the ubiquitinated proteins play a crucial role in the regulation of many cellular processes, including signal transduction (Paolini, Molfetta et al. 2002). THDPI did not show any significant effects on Syk kinase and proteasome activity. However, blocking the proteasome with MG132, a proteasome inhibitor, reversed the inhibitory effects of THDPI on phosphorylation of Syk and increased the amount of ubiquitinated Syk. These data indicate that THDPI does not affect Syk kinase and proteasome activity *in vitro*, but regulate Syk activation via ubiquitin-proteasome pathways.

Recently, several inhibitory factors regulating mast cell activation have been reported. Downstream of tyrosine kinase (Dok)-1 negatively regulates Ras by recruiting the Ras GTPase-activating protein, and MAPK phosphatase (MKP)-1 dephosphorylates and inactivate ERK and p38 MAPK in antigen-stimulated mast cells (Hiragun, Peng et al. 2005). Src-like adaptor protein (SLAP) is reported to regulate TCR signaling by associating with Zap70 and c-Cbl, Casitas B-lineage lymphoma (Hiragun, Peng et al. 2006). SLAP possesses Src homology (SH)2 and SH3 domains that are homologous to those in Src family kinases including Syk and Zap70 (Yamasaki and Saito 2004). It also reported that C-terminal region of SLAP interacts with Cbl, and C-terminal-truncated SLAP mutant disrupts the dimerization between SLAP proteins and loses its inhibitory effect on T cells-AP1 activity (Tang, Sawasdikosol et al. 1999). THDPI up-regulated SLAP expression and inhibitory effects on phosphorylated Syk by THDPI was blocked by small interfering RNA against SLAP in RBL-2H3 cells. Increased c-Cbl ligase activity in SLAP WT but not in SLAP ΔC also indicates that up-regulation of SLAP by THDPI might enhance E3 ligase activity and consequently phosphorylated Syk degradation. Altogether, These data support a mechanism of THDPI for regulating Syk activation in mast cells through the activation of the ubiquitin-proteasome pathway.

5. Conclusion

In conclusion, MFC and its active compounds, THDPI, inhibited FcεRI-mediated degranulation and the calcium influx. MFC also interfered with IgE-FcεRI interaction and decreased FcεRIβ mRNA expression. THDPI, however, did not show any significant effects on IgE-FcεRI interaction and FcεRI mRNA expression. It is possible that other minor compounds in MFC may be responsible for the inhibitory effects on the affinity of IgE to the receptors and the expression of FcεRIβ mRNA. Further study is required to identify other compounds in MFC responsible for these inhibitory effects. MFC and THDPI inhibited F-actin redistribution and downstream signaling by suppressing FcεRI-mediated Syk activation in mast cells (Figure 16). THDPI also up-regulated SLAP expression and regulating phosphorylated Syk levels in RBL-2H3 cells through the activation of the ubiquitin-proteasome pathway and provide a mechanism for Syk regulation involving c-Cbl ligase activity. These findings indicate that MFC and THDPI may have a potential therapeutic benefit for the controlling IgE-FcεRI-mediated mast cell activation in inflammatory and/or allergic process.

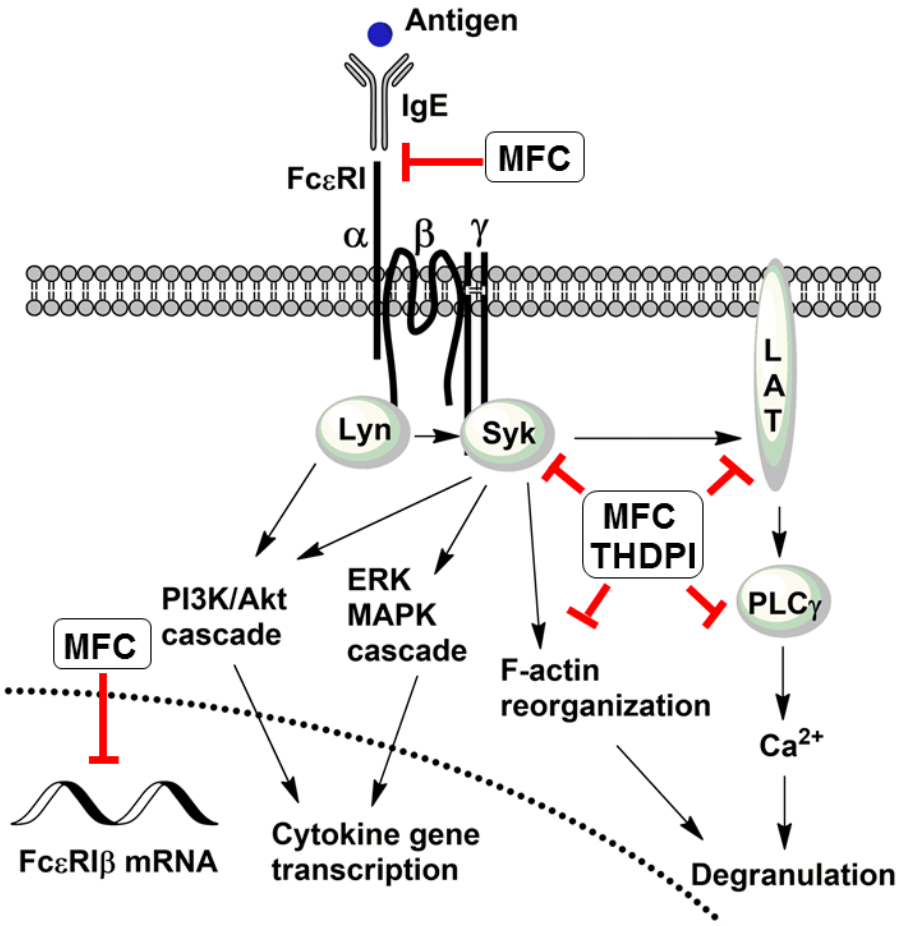


Figure 16. A scheme of the inhibitory effects of MFC and THDPI on FcεRI-mediated signaling in RBL-2H3 cells. MFC and THDPI inhibited mast cell degranulation, calcium influx, and F-actin redistribution. The phosphorylation of Syk, LAT, and PLCγ was inhibited by MFC and THDPI. In addition, MFC inhibited IgE-FcεRI interaction and FcεRIβ subunit mRNA expression.

Chapter 2

Effects of Magnolialide Isolated from the Leaves of *Laurus nobilis* L. (Lauraceae) on Immunoglobulin E-mediated Type I Hypersensitivity *in vitro*

1. Introduction

Type I hypersensitivity responses have been implicated in the cause of several diseases including allergic asthma and atopic dermatitis (Fuller, Morris et al. 1986). These responses are characteristically associated with immunoglobulin E (IgE) and mast cells play a crucial role in the development of IgE-mediated hypersensitivity reactions (Hu, Zhao et al. 2007). Mast cells possess a high-affinity IgE receptor (FcεRI) that binds to IgE (Onose, Xie et al. 2008). In type I hypersensitivity reactions, allergen interacts with mast cell-bound IgE and activates cells by cross-linking IgE-FcεRI complexes (Gounni 2006). The activation of mast cells following the engagement of IgE-FcεRI complex by antigens results in a process called degranulation (Matsubara, Masaki et al.

2004), and the activated mast cells release preformed molecules from its cytoplasmic granules, such as histamines, proteoglycans, serine proteases, and β-hexosaminidase, which are capable of inducing bronchoconstriction, mucus secretion, and mucosal edema, all features of asthma (Bradding, Walls et al. 2006). Degranulation of mast cells with the resulting release of histamine and β-hexosaminidase elicits the production of cytokines including interleukin (IL)-4, which activates pre-T helper (pre-Th) cells and transforms into T helper 2 (Th2) cells (Biedermann, Rocken et al. 2004), and the overexpression of the IL-4 affects Th2 differentiation and IgE class switch. (Guo, Qiao et al. 2004). IL-4 levels are elevated in patients with severe asthma and atopic dermatitis, and this increase in the IL-4 levels also correlates with the severity of these diseases (Cho, Stanciu et al. 2005, Dhar, Malakar et al. 2005). IL-5, one of the main pro-inflammatory mediators produced by Th2 cells, stimulates eosinophilic inflammation and mucus production (Sampson 2001) and induces early B cell proliferation and immunoglobulin secretion (Togawa, Kiniwa et al. 2001). IL-5 acted as a stimulator of immunoglobulin productions from activated B cells and the proliferation of eosinophils, and its synthesis is up-regulated by mast cells activated in type I hypersensitivity allergic reactions (Sampson 2001, Kouro and Takatsu 2009).

The rat basophilic leukemia mast cell line (RBL-2H3) has been commonly used as an *in vitro* model for IgE-mediated responses in mast cells (Suzuki, Yoshimaru et al. 2001). RBL-2H3 cells produce high-affinity FcεRI receptors for IgE on the cell-surface membrane and aggregation of the IgE-FcεRI complex caused by antigens activates various cascades of intracellular events that result in mast cell degranulation and the consequent release of chemical mediators of IgE-mediated responses (Qu, Miah et al. 2005). Y16 cell is characterized as an early B cell and has been used for an IL-5-dependent early B cell proliferation assay (Sato, Katagiri et al. 1994).

Laurus nobilis L. (Lauraceae), commonly known as Bay, Sweet Bay, True Laurel, or Roman Laurel, is widely spread in the Mediterranean area and Europe (Dall'Acqua, Viola et al. 2006), and leaves of this plant are used as a folk medicine in the treatment of arthritis (Lev and Amar 2000), rheumatic pains (Bruni, Ballero et al. 1997), skin inflammation (Pieroni, Quave et al. 2004), and asthma (Loi, Poli et al. 2004). Previously, phytochemical studies have led to isolation of several classes of secondary metabolites of *Laurus nobilis* L. including glycosylated flavonoids (Fiorini, David et al. 1998), megastigmane and phenolic components (De Marino, Borbone et al. 2004), monoterpenes, and sesquiterpenes (Dall'Acqua, Viola et al. 2006, Julianti, Jang et al. 2012). Sesquiterpene lactones, most diverse metabolites of bay leaf,

have been investigated for various pharmacological effects such as neuroprotective (Ham, Shin et al. 2011), cytotoxic (Barla, Topcu et al. 2007), and anti-inflammatory activities (Hall, Lee et al. 1979), but little has been investigated to validate the scientific evaluation of *Laurus nobilis* L. for type I hypersensitivity disorders such as atopic dermatitis and asthma. In this study, the effects of sesquiterpenes isolated from the leaves of *Laurus nobilis* L. especially focusing on mast cell degranulation, IL-4 production in RBL-2H3 cells and IL-5-dependent B cell proliferation were investigated.

2. Materials and Methods

2.1. Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 medium, monoclonal anti-dinitrophenyl (DNP) IgE mouse antibody, 4-nitrophenyl N-acetyl-β-glucosaminide, DNP-conjugated human serum albumin (HSA), and 2-mercaptoethanol, doxorubicin hydrochloride (purity ≥ 98%), cromoglycate (purity ≥ 95%), ketotifen fumarate salt (purity ≥ 99%), and tyrphostin AG 490 (purity ≥ 98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Invitrogen (Carlsbad, CA, USA). *Prime* RT-PCR premix™ was purchased from Genet Bio (Seoul, Korea), and easy-BLUE™ total RNA extraction kits were purchased from iNtRON Biotechnology (Kyunggi, Korea).

2.2. Plant material

The leaves of *Laurus nobilis* L. were purchased from Trukey (Orege Forest Agricultural and Food Products Foreign Trade Ltd.) in August 2007, and identified by Dr. Yeong-han Kwon of Korea National Arboretum. A voucher

specimen (NPRI-Q003) has been deposited in Natural Products Research Institute, Seoul National University.

2.3. Extraction and isolation

The preparation of extracts from leaves of *Laurus nobilis* L. was described in a previous study (Julianti, Jang et al. 2012). The isolation and identification of seven compounds, magnolialide (1), santamarine (2), reynosin (3) baynol C (4) 11,13-dehydrosantonin (5),(3a*S*,5a*R*,6*R*,9*S*,9a*S*,9b*S*)-6,9-dihydroxy-5a,9-dimethyl-3-methylidene-3a,4,5,6,7,8,9a,9boctahydrobenzo[g][1]benzofuran-2-one (DDMO) (**6**), and lucentolide (**7**) were described in a previous study (Lee, Lee et al. 2013).

2.4. Cell culture

Rat basophilic leukemia (RBL-2H3) cells (ATCC No. CRL-2256) were cultured at 37°C, 5% CO_2 in DMEM supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin.

2.5. Cell viability test

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was conducted to examine cell viability (Twentyman and Luscombe 1987). RBL-2H3 cells (5 x 10^5 cells/mL, 100uL/well) were plated into a 96-well plate. After 24 hrs of incubation, cells were incubated with various concentrations of 7 isolated compounds for 3 hrs and medium was replaced with MTT dissolved in phenol-red free medium (250 μg/mL) and incubated at 37°C for 4 hrs. The medium was carefully discarded and formazan was resuspended in 200 μL of dimethyl sulfoxide (DMSO). The absorbance was measured at 595 nm using a microplate reader. Values measured from untreated cells were considered to represent 100 % viability.

2.6. Measurement of β-hexosaminidase release from RBL-2H3 cells

Release of β-hexosamindase was measured based on the previously described method (Mastuda, Morikawa et al. 2002). Briefly, RBL-2H3 cells (5 x 10^5 cells/mL, 100uL/well) seeded in 96-well plates were incubated with monoclonal mouse anti-dinitrophenyl IgE antibody (anti-DNP IgE) (1 μg/mL) for 24 hrs at 37°C. The IgE-sensitized RBL-2H3 cells were washed twice with

PBS buffer to remove excess IgE in medium. After washing, the cells were pre-incubated with each isolated compound in 100 μL of fresh medium at 37°C for 20 mins followed by 400 ng/mL of multivalent antigen, DNP-HSA for 1hr. Supernatants (40 μL) were transferred to a 96-well plate and incubated for 1.5 hrs at 37°C with 50 μL of substrate solution (5 mM of 4-nitrophenyl N-acetyl-β-D-glucosaminide in 50 mM of citric acid buffer, pH 4.5). The reactions were quenched by the addition of 50 μL of stop solution (0.5 M Na_2CO_3/$NaHCO_3$, pH 10.0) and the absorbance at 405 nm was measured. Activity values measured from cells treated with anti-DNP IgE and DNP-HSA were considered to represent 100 % of degranulation.

2.7. Measurement of IL-4 cytokine release from RBL-2H3 cells

RBL-2H3 cells (5 x 10^5 cells/mL, 300uL/well) were sensitized for 24 hrs with 1 μg/mL of anti-DNP IgE in 48-well plates. Later, the IgE-sensitized RBL-2H3 cells were treated with each isolated compound for 3 hrs followed by DNP-HSA for an additional 2 hrs. The amount of IL-4 released in culture medium was measured using Rat IL-4 Platinum ELISA™ kit (eBioscience, USA), according to the manufacturer's instruction.

2.8. Measurement of IL-4 mRNA expression in RBL-2H3 cells by reverse transcription-polymerase chain reaction (RT-PCR)

RBL-2H3 cells (5 x 105 cells/mL, 500uL/well) were cultured for 24 hrs in 24-well plates with anti-DNP IgE (1 μg/mL). The RBL-2H3 cells were washed two times with PBS buffer and replaced with fresh media, then incubated with each isolated compound for 3 hrs at 37°C followed by DNP-HSA at 400 ng/mL for an additional 1 hr. Total RNA was isolated with easy-BLUETM (iNtRON Biotechnology, Korea), according to the manufacturer's instruction. cDNA synthesis was performed from 500 ng of total RNA by using ONE-STEP RT-PCR PreMix kit (iNtRoN Biotechnology, Korea). The following primer pairs were synthesized. (Bioneer, Korea).

IL-4, 5'-GGGTGCTTCGCAAATTTTACTT-3' (sense)
5'-ACCGAGAACCCC AGACTTGTT-3' (antisense)
β-actin, 5'-TCTGTGTGGATTGGTGGCTCTA-3' (sense)
5'-CTGCTTGCTGATCCACATCTG-3' (antisense)

The denaturation, annealing, extension, and cycle conditions were as follows: IL-4: 94°C for 45 s, 58°C for 45 s, 72°C for 45 s and 34 cycles; β-actin: 94°C for 45 s, 55°C for 45 s, 72°C for 45 s and 30 cycles. The PCR reaction was performed with a 2720 Thermal Cycler (Applied Biosystems, USA). Aliquots

of the PCR reactions were electrophoresed in 1.5 % (w/v) agarose gels stained with SYBR Safe DNA gel stain (Invitrogen, USA). DNA bands were visualized with LAS 1000plus (FUJIFILM, Japan).

2.9. Y16 early B cell proliferation assay

Y16 cells (Takaki, Tominaga et al. 1990) (5 x 10^5 cells /mL, 100uL/well) were plated to a 96-well microplate with RPMI-1640 supplemented with 24 mM $NaHCO_3$, 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μM 2-mercaptoethanol, and 10 U/mL of IL-5 (Prospec, Isreal). Cells were treated with isolated compounds or a positive control compound, tyrphostin AG490 (Sigma-Aldrich, USA), for 48 hrs. MTT assay was performed to measure cell proliferation by measuring the absorbance at 540 nm using a microplate reader.

2.10. Statistical analysis

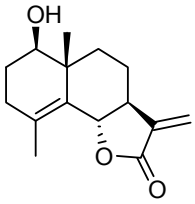
All data were presented as the mean ± S.D. of at least three independent experiments performed in triplicate. Statistical significance was determined using GraphPad Prism (GraphPad Software, USA). The differences among groups were evaluated by one-way analysis of variance (ANOVA) with

Bonferroni's multiple comparison method. *P* value less than 0.05 was considered to be statistically significant.

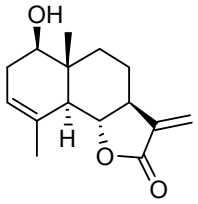
3. Results

3.1. Structures of isolated compounds and their cytotoxicity

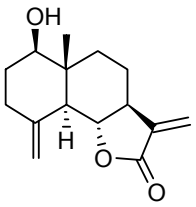
The combined MeOH and CH_2Cl_2 extract of leaves of *Laurus nobils* L. was subfractioned and seven isolates (Figure 17) were obtained from $CHCl_3$ subfraction. Cytotoxic effects of 7 compounds were determined after 24 hours of treatment in RBL-2H3 cells using the MTT assay at various concentrations up to 100 μM (Figure 18). In this experiment, baynol C and lucentolide revealed relatively strong cytotoxic effects at a concentration of 100 μM (70.4% survival and 79.2% survival, respectively), whereas magnolialide revealed a relatively weak cytotoxic effect (91.1% survival at 100 μM), and the other compounds showed a little or non-cytotoxic (>95.2% survival at 100 μM). A positive control compound, doxorubicin, revealed a cytotoxic effect with an IC_{50} value of 29.2 μM.



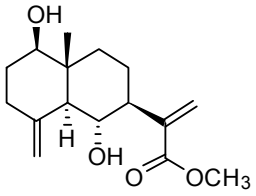
magnolialide (**1**)



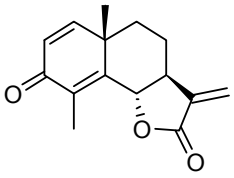
santamarine (**2**)



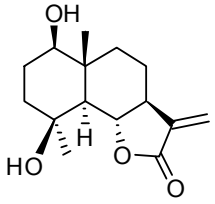
reynosin (**3**)



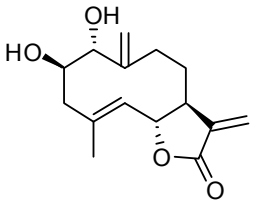
baynol C (**4**)



11,13-dehydrosantonin (**5**)



(3a*S*,5a*R*,6*R*,9*S*,9a*S*,9b*S*)-6,9-dihydroxy-5a,9-dimethyl-3-methylidene-3a,4,5,6,7,8,9a,9b-octahydrobenzo[g][1]benzofuran-2-one (**6**)



lucentolide (**7**)

Figure 17. Chemical structures of isolated compounds from *Laurus nobilis*.

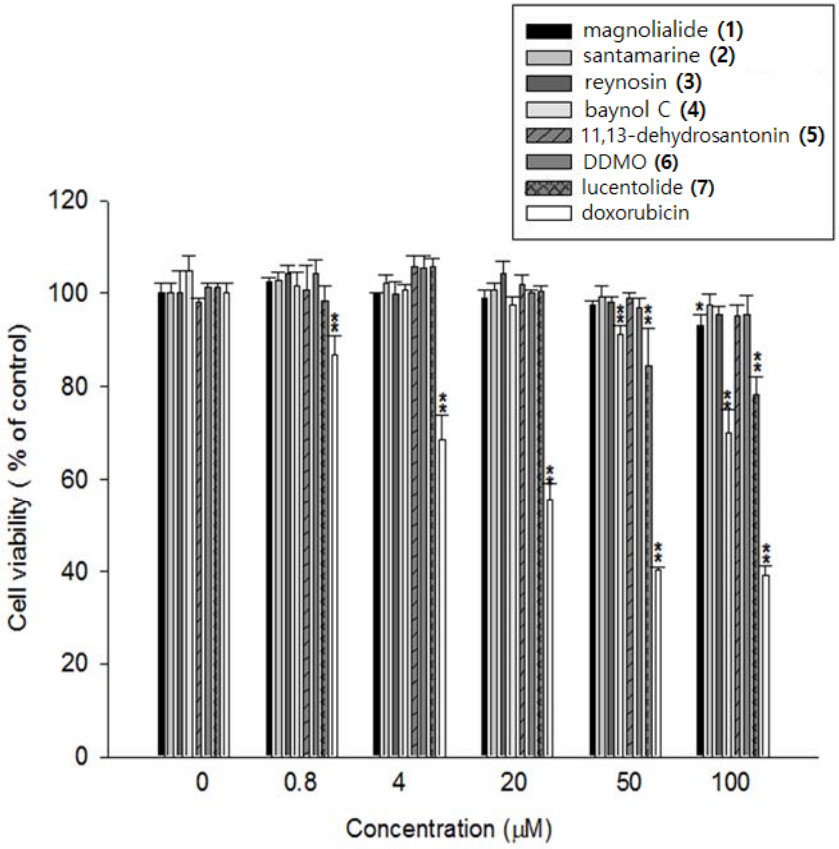


Figure 18. Cytotoxic effects of compounds isolated from *Laurus nobilis* on RBL-2H3 cells. RBL-2H3 cells were treated with each isolated compound or doxorubicin (a positive control) for 24 hrs and cell viability was determined by MTT assay. All data were obtained from three independent experiments performed in triplicate. Data represent the mean ± S.D. of three independent experiments, $^{*}p < 0.05$, $^{**}p < 0.01$, compared with vehicle treatment group.

3.2. Effect of magnolialide on mast cell degranulation

The degranulation of RBL-2H3 mast cells, after sensitization with anti-DNP IgE and reaction with DNP-HSA, was evaluated by measuring the activity of β-hexosaminidase released. Treatment with magnolialide inhibited the release of β-hexosaminidase with an IC_{50} value of 20.2 μM, and mast cell stabilizers, cromoglycate and ketotifen, used as positive control compounds, revealed IC_{50} values of 74.3 and 58.4 μM, respectively (Figure 19).

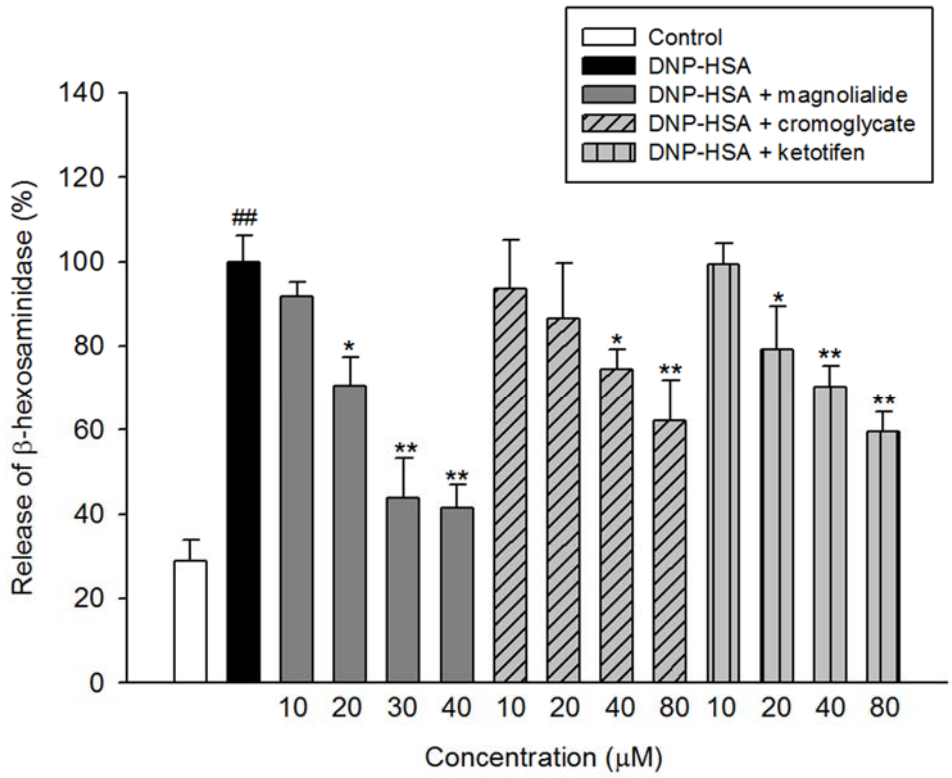


Figure 19. Inhibitory effects of magnolialide on β-hexosaminidase release from RBL-2H3 cells. Values measured from cells treated with anti-DNP IgE and DNP-HSA were considered to represent 100 % of degranulation. Cells were treated with magnolialide or positive control compounds (cromoglycate and ketotifen) for 20 min followed by DNP-HSA for an hour. Data represent the mean ± S.D. of three independent experiments; $^{\#\#}p < 0.01$, compared with control group (anti-DNP IgE treatment alone); $^{*}p < 0.05$, $^{**}p < 0.01$, compared with anti-DNP IgE and DNP-HSA treatment.

3.3. Effect of magnolialide on IL-4 production

The effect of magnolialide on the release of IL-4 was determined by measuring the IL-4 concentration in the cultured medium by using the ELISA kit. Magnolialide inhibited the IL-4 release with an IC_{50} value of 18.1 μM; the positive control compounds, cromoglycate and ketotifen, also inhibited IL-4 release with IC_{50} values of 7.4 and 15.2 μM, respectively **(**Figure 20**).** In addition, the effect on the expression of IL-4 mRNA was investigated. Magnolialide reduced the IL-4 mRNA expression with an IC_{50} value of 15.7 μM, cromoglycate with an IC_{50} value of 8.1 μM, and ketotifen with an IC_{50} value of 19.4 μM (Figure 21). Other six isolated compounds, santamarine, reynosin, baynol C, 11,13-dehydrosantonin, (3a*S*,5a*R*,6*R*,9*S*,9a*S*,9b*S*)-6,9-dihydroxy-5a,9-dimethyl-3-methylidene-3a,4,5,6,7,8,9a,9b-octahydrobenzo[g][1]benzofuran-2-one, and lucentolide did not show any significant inhibitory effects at concentrations tested.

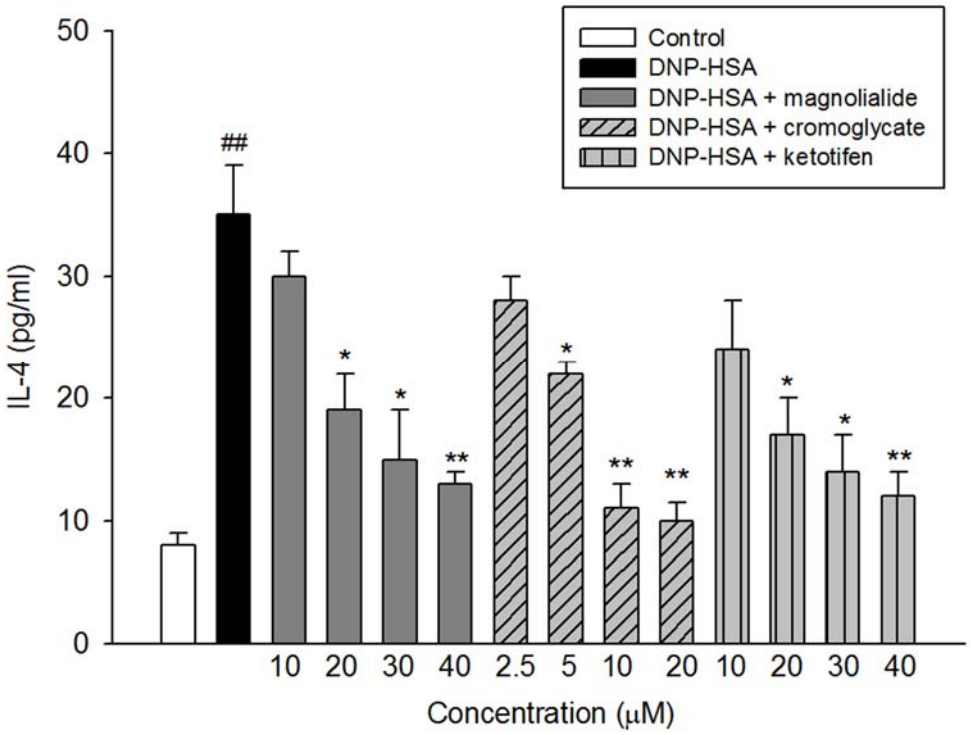


Figure 20. Inhibitory effect of magnolialide on IL-4 release from RBL-2H3 cells. Cells were treated with magnolialide or positive control compounds (cromoglycate and ketotifen) for 3 hrs followed by DNP-HSA for 2 hrs. The release of IL-4 was measured by ELISA as described in materials and methods. Data represent the mean $\pm$ S.D. of three independent experiments; $^{\#\#}p < 0.05$, compared with control group (anti-DNP IgE treatment alone); $^{*}p < 0.05$, $^{**}p < 0.01$, compared with anti-DNP IgE and DNP-HSA treatment.

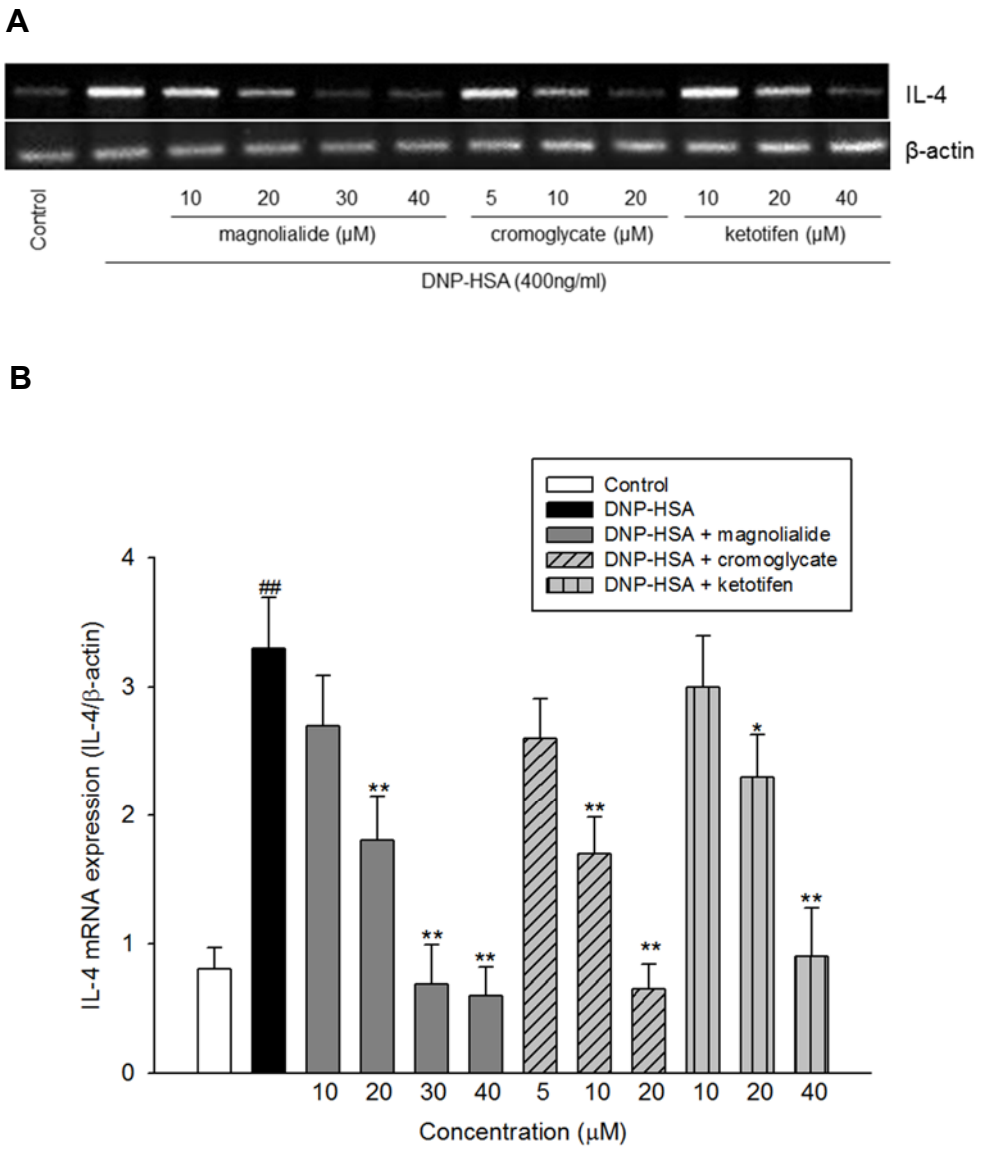


Figure 21. Inhibitory effect of magnolialide on the expression of IL-4 mRNA in RBL-2H3 cells. Cells were treated with magnolialide or positive control compounds (cromoglycate or ketotifen) for 3 hr followed by DNP-

HSA for 1 hr. (**A**) Inhibitory effects of magnolialide, cromoglycate, and ketotifen on the expression of IL-4 mRNA in RBL-2H3 cells. (**B)** Quantitative representation of expression of IL-4 and β-actin mRNA using Image J software. Data represent the mean ± S.D. of three independent experiments; $^{\#\#}p < 0.01$, compared with control group (anti-DNP IgE treatment alone); $^{*}p < 0.05$, $^{**}p < 0.01$, compared with anti-DNP IgE and DNP-HSA treatment.

3.4. Effect of magnolialide on Y16 cell proliferation

In addition, the inhibitory effect of magnolialide on IL-5-stimulated Y16 cell proliferation was examined and inhibition was also observed in a dose dependent manner (Figure 22). The IC_{50} values of magnolialide and of the positive control compound tyrphostin AG490 were 18.4 μM and 24.9 μM, respectively.

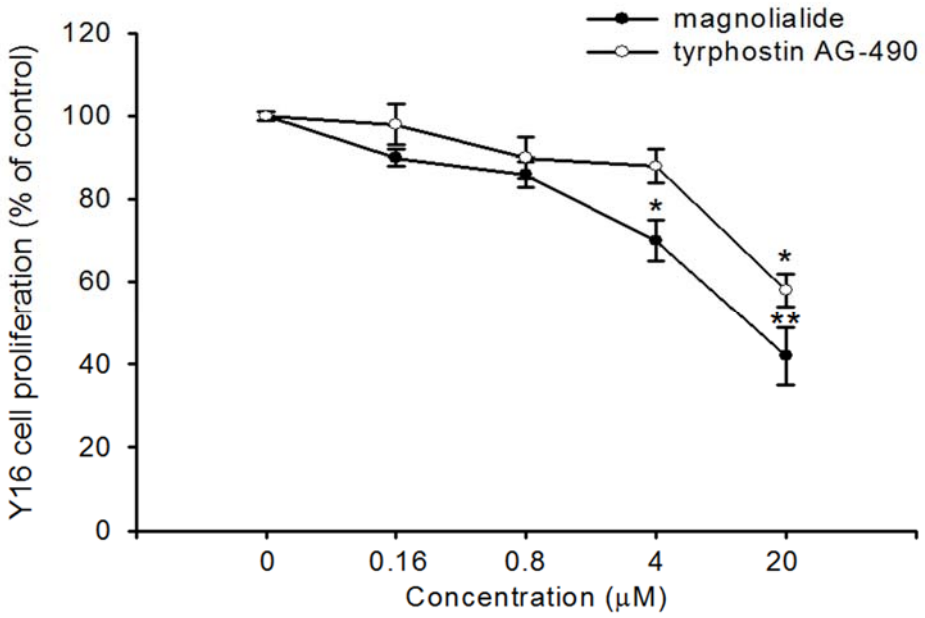


Figure 22. Inhibitory effects of magnolialide on IL-5-dependent proliferation of Y16 cells. Y16 cells were cultured with IL-5 with or without magnolialide or tryphostin AG 490 for 48 hrs. Cell growth was measured by MTT assay. IL-5 treated cells with vechicle treatment were used as repsesenting 100% cell proliferation. Data represent the means ± S.D. of three independent experiments; $^{*}p < 0.05$; $^{**}p < 0.01$, compared with vehicle treatment group.

4. Discussion

The mast cell has been reported to associate with skin inflammation (Harvima and Nilsson 2011) and asthma (Carroll, Mutavdzic et al. 2002), and mast cell degranulation along with several cytokines produced from activated mast cells is considered to play pivotal roles in these responses (Bradding, Roberts et al. 1994). In this study, we investigated several isolated compounds from the leaves of *Laurus nobilis* L. which was traditionally used for skin disease and asthma therapy, and especially focused on their activity against IgE-mediated hypersensitivity responses from mast cells. First, we isolated seven sesquiterpene lactones from the leaves of *Laurus nobilis* L., and found that magnolialide significantly inhibited IgE-mediated degranulation from RBL-2H3 mast cells. No significant toxicity was observed at the range of concentrations tested in this experiment. The inhibition of mast cell degranulation was compared with cromoteglycate (Theoharides, Sieghart et al. 1980) and ketotifen (Juanola, Giralt et al. 1998), mast cell stabilizers. The inhibitory effects of these two agents were relatively weak compared to magnolialide (Table 3).

Table 3. Inhibitory effects of compounds from *Laurus nobilis* L. on the β-hexosaminidase release and IL-4 production from antigen-induced RBL-2H3 cells, and IL-5-dependent proliferation of Y16 cells.

Compound	β-hexosaminidase release (IC_{50} ± SD, μM)	IL-4 production (IC_{50} ± SD, μM)		IL-5-dependent proliferation (IC_{50} ± SD, μM)
		release	mRNA expression	
magnolialide (**1**)	20.2 ± 4.9	18.1 ± 4.1	15.7 ± 3.1	18.4 ± 4.2
santamarine (**2**)	>100	>100	>100	>100
reynosin (**3**)	>100	>100	>100	>100
baynol C [b] (**4**)	>20	>20	>20	>20
11,13-dehydrosantonin (**5**)	>100	>100	>100	>100
DDMO [a] (**6**)	>100	>100	>100	>100
lucentolide [b] (**7**)	>20	>20	>20	>20
cromoglycate (control)	74.3 ± 5.5	7.4 ± 3.7	8.1 ± 4.0	n.d
ketotifen (control)	58.4 ± 3.3	15.2 ± 4.2	19.4 ± 4.9	n.d.
tyrphostin AG490 (control)	n.d.	n.d.	n.d.	24.9 ± 3.9

The IC_{50} values were determined in a semilogarithmic graph depicting the relationship between at least 4 different concentrations of compounds and the each percentage of inhibition.

[a] DDMO : (3a*S*,5a*R*,6*R*,9*S*,9a*S*,9b*S*)-6,9-dihydroxy-5a,9-dimethyl-3-methylidene-3a,4,5,6,7,8,9a,9b-octahydrobenzo[g][1]benzofuran-2-one

[b] Cytotoxic at a concentration higher than 20 μM

n.d. : not determined

Besides degranulation during IgE-mediated reactions, mast cells also concomitantly synthesize and release a variety of cytokines, including IL-4, IL-5, IL-13, and tumor necrosis factor-α, during the late-phase reaction (Metcalfe, Baram et al. 1997). Among those cytokines, IL-4 derived from mast cells is known to be important in the mucosal inflammatory responses and in the development and amplification of IgE-dependent allergic responses (Bradding, Roberts et al. 1994). The reduction of IL-4 production by cromoglycate and ketotifen in IgE-mediated RBL-2H3 cells has been demonstrated (Matsubara, Masaki et al. 2004). In the present study, IL-4 release and IL-4 mRNA expression levels in RBL-2H3 cells were significantly inhibited by magnolialide. In comparison with cromoglycate and ketotifen, magnolialide was less potent than cromoglycate but it has a similar inhibitory effect to ketotifen (Table 3). Thus, magnolialide might contribute to alleviate the inflammatory and late allergic symptoms by suppressing the expression and production of IL-4 in mast cells.

Besides IL-4, it was reported that IL-5, another pro-inflammatory cytokine, is produced by Th2 cells and activates mast cells (Bradding, Roberts et al. 1994). The mast cell-derived IL-5 induces early B cell proliferation and subsequently increases in immunoglobulin secretion (Yamaguchi, Hayashi et al. 1988). In this study, treatment with magnolialide significantly inhibited the

proliferation of IL-5-dependent Y16 early B cells, and the inhibitory effect was 1.3-times more potent than that of tyrphostin AG490, a control compound with a specific inhibitory effect against Janus kinase 2 (JAK2) which is critical for the anti-apoptotic effect induced by IL-5 (Pazdrak, Olszewska-Pazdrak et al. 1998). These results demonstrated that magnolialide might be able to control IgE-mediated hypersensitivity reactions by not only suppressing the early phases of mast cell activation, but also suppressing subsequent IgE amplification by the IL-5-dependent proliferation of B cells.

5. Conclusion

The present study demonstrated for the first time that magnolialide isolated from the leaves of *Laurus nobilis* L. effectively inhibited the mast cell degranulation and IL-4 production in IgE-involved responses in RBL-2H3 mast cells, and suppressed the proliferation of IL-5-dependent Y16 early B cells. Some sesquiterpene lactones such as parthenin, frullanolide, and alantolactone are reported to cause allergic contact dermatitis, which is a type-IV T-cell-mediated reaction (Mitchell, Fritig et al. 1970). However, several studies have reported the possible therapeutic effects of sesquiterpene lactones for a type-I IgE-mediated-hypersensitivity. An extract of the plant feverfew (*Tanancetum parthenium*), a rich source of sesquiterpene lactones, inhibited anti-IgE-induced histamine release from rat peritoneal mast cells (Hayes and Foreman 1987). Also, sesquiterpene lactones such as isobutyroylplenolin and senecioylplenolin from *Centipeda minima* exhibited significant anti-allergic activity in passive cutaneous anaphylaxis (PCA) test with p.o. administration (Wu, Chun et al. 1991). It was also reported that parthenolide, a sesquiterpene lactone, suppressed IL-4 production in peripheral blood T cells by blocking NF-κB binding to IL-4 promoter regulatory regions, suggesting a treatment of IL-4-mediated allergic inflammation (Li-Weber, Giaisi et al. 2002). In

conclusion, our results suggest that magnolialide might have therapeutic potential for a type-I IgE-mediated allergic inflammatory disorder such as asthma and atopic dermatitis through a mast cell stabilizing effect and inhibition of IL-4 production and proliferation of IL-5-dependent early B cells, although further investigation of magnolialide involved in IL-4-mediated and IL-5-mediated signal transduction processes is warranted.

References

Bajpai, V. K., A. Sharma and K. H. Baek (2013). "Antibacterial mode of action of Cudrania tricuspidata fruit essential oil, affecting membrane permeability and surface characteristics of food-borne pathogens." *Food Control* **32**(2): 582-590.

Barla, A., G. Topcu, S. Oksuz, G. Tumen and D. G. I. Kingston (2007). "Identification of cytotoxic sesquiterpenes from Laurus nobilis L." *Food Chem* **104**(4): 1478-1484.

Biedermann, T., M. Rocken and J. M. Carballido (2004). "TH1 and TH2 lymphocyte development and regulation of TH cell-mediated immune responses of the skin." *J Investig Dermatol Symp Proc* **9**(1): 5-14.

Bradding, P., J. A. Roberts, K. M. Britten, S. Montefort, R. Djukanovic, R. Mueller, C. H. Heusser, P. H. Howarth and S. T. Holgate (1994). "Interleukin-4, -5, and -6 and tumor necrosis factor-alpha in normal and asthmatic airways: evidence for the human mast cell as a source of these cytokines." *Am J Respir Cell Mol Biol* **10**(5): 471-480.

Bradding, P., A. F. Walls and S. T. Holgate (2006). "The role of the mast cell in the pathophysiology of asthma." *J Allergy Clin Immunol* **117**(6): 1277-1284.

Bruni, A., M. Ballero and F. Poli (1997). "Quantitative ethnopharmacological study of the Campidano Valley and Urzulei district, Sardinia, Italy." *J Ethnopharmacol* **57**(2): 97-124.

Carroll, N. G., S. Mutavdzic and A. L. James (2002). "Distribution and degranulation of airway mast cells in normal and asthmatic subjects." *Eur Respir J* **19**(5): 879-885.

Cho, S. H., L. A. Stanciu, S. T. Holgate and S. L. Johnston (2005). "Increased interleukin-4, interleukin-5, and interferon-gamma in airway CD4+ and CD8+ T cells in atopic asthma." *Am J Respir Crit Care Med* **171**(3): 224-230.

Cox, D., P. Chang, T. Kurosaki and S. Greenberg (1996). "Syk tyrosine kinase is required for immunoreceptor tyrosine activation motif-dependent actin assembly." *J Biol Chem* **271**(28): 16597-16602.

Dall'Acqua, S., G. Viola, M. Giorgetti, M. C. Loi and G. Innocenti (2006). "Two new sesquiterpene lactones from the leaves of Laurus nobilis." *Chem Pharm Bull (Tokyo)* **54**(8): 1187-1189.

de Castro, R. O. (2011). "Regulation and function of syk tyrosine kinase in mast cell signaling and beyond." *J Signal Transduct* **2011**: 507291.

De Marino, S., N. Borbone, F. Zollo, A. Ianaro, P. Di Meglio and M. Iorizzi (2004). "Megastigmane and phenolic components from Laurus nobilis L. leaves and their inhibitory effects on nitric oxide production." *J Agric Food Chem* **52**(25): 7525-7531.

Devivar, A. R. and H. Jimenez (1965). "Structure of Santamarine a New Sesquiterpene Lactone." *Tetrahedron* **21**(7): 1741-&.

Dhar, S., R. Malakar, S. Chattopadhyay, R. Banerjee and A. Ghosh (2005). "Correlation of the severity of atopic dermatitis with absolute eosinophil counts in peripheral blood and serum IgE levels." *Indian J Dermatol Venereol Leprol* **71**(4): 246-249.

Dombrowicz, D., V. Flamand, K. K. Brigman, B. H. Koller and J. P. Kinet (1993). "Abolition of anaphylaxis by targeted disruption of the high affinity immunoglobulin E receptor alpha chain gene." *Cell* **75**(5): 969-976.

Elferaly, F. S., Y. M. Chan and D. A. Benigni (1979). "Magnolialide - Novel Eudesmanolide from the Root Bark of Magnolia-Grandiflora." *Phytochemistry* **18**(5): 881-882.

Fiorini, C., B. David, I. Fouraste and J. Vercauteren (1998). "Acylated kaempferol glycosides from Laurus nobilis leaves." *Phytochemistry* **47**(5): 821-824.

Fraga, B. M. (2002). "Natural sesquiterpenoids." *Nat Prod Rep* **19**(5): 650-672.

Fuller, R. W., P. K. Morris, R. Richmond, D. Sykes, I. M. Varndell, D. M. Kemeny, P. J. Cole, C. T. Dollery and J. MacDermot (1986). "Immunoglobulin E-dependent stimulation of human alveolar macrophages: significance in type 1 hypersensitivity." *Clin Exp Immunol* **65**(2): 416-426.

Gounni, A. S. (2006). "The high-affinity IgE receptor (FcepsilonRI): a critical regulator of airway smooth muscle cells?" *Am J Physiol Lung Cell Mol Physiol* **291**(3): L312-321.

Gu, W., K. Q. Han, Y. H. Su, X. Q. Huang and C. Q. Ling (2007). "[Inhibition action of bufalin on human transplanted hepatocellular tumor and its effects on expressions of Bcl-2 and Bax proteins in nude mice]." *Zhong Xi Yi Jie He Xue Bao* **5**(2): 155-159.

Guo, H., Z. Qiao, L. Zhu, H. Wang, L. Su, Y. Lu, Y. Cui, B. Jiang, Q. Zhu and L. Xu (2004). "Th1/Th2 cytokine profiles and their relationship to clinical features in patients following nonmyeloablative allogeneic stem cell transplantation." *Am J Hematol* **75**(2): 78-83.

Hall, I. H., K. H. Lee, C. O. Starnes, Y. Sumida, R. Y. Wu, T. G. Waddell, J. W. Cochran and K. G. Gerhart (1979). "Anti-inflammatory activity of sesquiterpene lactones and related compounds." *J Pharm Sci* **68**(5): 537-542.

Ham, A., J. Shin, K. B. Oh, S. J. Lee, K. W. Nam, U. Koo, K. H. Kim and W. Mar (2011). "Neuroprotective Effect of the n-Hexane Extracts of Laurus

nobilis L. in Models of Parkinson's Disease." *Biomol Ther* **19**(1): 118-125.

Han, X. H., S. S. Hong, J. S. Hwang, S. H. Jeong, J. H. Hwang, M. H. Lee, M. K. Lee, D. Lee, J. S. Ro and B. Y. Hwang (2005). "Monoamine oxidase inhibitory constituents from the fruits of Cudrania tricuspidata." *Arch Pharm Res* **28**(12): 1324-1327.

Han, X. H., S. S. Hong, Q. Jin, D. Li, H. K. Kim, J. Lee, S. H. Kwon, D. Lee, C. K. Lee, M. K. Lee and B. Y. Hwang (2009). "Prenylated and benzylated flavonoids from the fruits of Cudrania tricuspidata." *J Nat Prod* **72**(1): 164-167.

Harvima, I. T. and G. Nilsson (2011). "Mast Cells as Regulators of Skin Inflammation and Immunity." *Acta Derm-Venereol* **91**(6): 644-650.

Hayes, N. A. and J. C. Foreman (1987). "The activity of compounds extracted from feverfew on histamine release from rat mast cells." *J Pharm Pharmacol* **39**(6): 466-470.

Hiep, N. T., J. Kwon, D. W. Kim, B. Y. Hwang, H. J. Lee, W. Mar and D. Lee

(2014). "Isoflavones with neuroprotective activities from fruits of Cudrania tricuspidata." *Phytochemistry* **77**(8): 1893-1901.

Hiragun, T., Z. Peng and M. A. Beaven (2005) "Dexamethasone up-regulates the inhibitory adaptor protein Dok-1 and suppresses downstream activation of the mitogen-activated protein kinase pathway in antigen-stimulated RBL-2H3 mast cells" *Mol Pharmacol* **67**(3): 598-603.

Hiragun, T., Z. Peng and M. A. Beaven (2006) "Cutting edge: dexamethasone negatively regulates syk in mast cells by up-regulating src-like adaptor protein" *J Immunol* **177**: 2047-2050.

Hu, Z. Q., W. H. Zhao and T. Shimamura (2007). "Regulation of mast cell development by inflammatory factors." *Curr Med Chem* **14**(28): 3044-3050.

Huang, F., K. Yamaki, X. Tong, L. Fu, R. Zhang, Y. Cai, R. Yanagisawa, K. Inoue, H. Takano and S. Yoshino (2008). "Inhibition of the antigen-induced activation of RBL-2H3 cells by sinomenine." *Int Immunopharmacol* **8**(3): 502-507.

Iida, M., S. Wakuri, S. Mineki, K. Nishitani and K. Yamakawa (1993). "Studies on Microbiological Transformation .28. Microbial Hydroxylation of 11,13-Dehydrosantonin by Aspergillus-Niger." *J Ferment Bioeng* **76**(4): 296-299.

Ishida, M., K. Nishi, H. Watanabe and T. Sugahara (2013). "Inhibitory effect of aqueous spinach extract on degranulation of RBL-2H3 cells." *Food Chem* **136**(2): 322-327.

Itoh, T., M. Ninomiya, M. Yasuda, K. Koshikawa, Y. Deyashiki, Y. Nozawa, Y. Akao and M. Koketsu (2009). "Inhibitory effects of flavonoids isolated from Fragaria ananassa Duch on IgE-mediated degranulation in rat basophilic leukemia RBL-2H3." *Bioorg Med Chem* **17**(15): 5374-5379.

Jeong, C. H., G. N. Choi, J. H. Kim, J. H. Kwak, H. R. Jeong, D. O. Kim and H. J. Heo (2010). "Protective Effects of Aqueous Extract from Cudrania tricuspidata on Oxidative Stress-induced Neurotoxicity." *Food Sci Biotechnol* **19**(4): 1113-1117.

Juanola, C., M. Giralt, M. Jimenez, M. Mourelle and P. Vergara (1998).

"Mucosal mast cells are involved in CCK disruption of MMC in the rat intestine." *Am J Physiol* **275**(1 Pt 1): G63-67.

Julianti, E., K. H. Jang, S. Lee, D. Lee, W. Mar, K. B. Oh and J. Shin (2012). "Sesquiterpenes from the leaves of Laurus nobilis L." *Phytochemistry* **80**: 70-76.

Kim, M., S. J. Lim, S. W. Kang, B. H. Um and C. W. Nho (2014). "Aceriphyllum rossii extract and its active compounds, quercetin and kaempferol inhibit IgE-mediated mast cell activation and passive cutaneous anaphylaxis." *J Agric Food Chem* **62**(17): 3750-3758.

Knol, E. F. (2006). "Requirements for effective IgE cross-linking on mast cells and basophils." *Mol Nutr Food Res* **50**(7): 620-624.

Kouro, T. and K. Takatsu (2009). "IL-5- and eosinophil-mediated inflammation: from discovery to therapy." *Int Immunol* **21**(12): 1303-1309.

Kraft, S. and J. P. Kinet (2007). "New developments in FcepsilonRI regulation, function and inhibition." *Nat Rev Immunol* **7**(5): 365-378.

Lee, H., H. Ha, J. K. Lee, C. S. Seo, N. H. Lee, D. Y. Jung, S. J. Park and H. K. Shin (2012). "The fruits of Cudrania tricuspidata suppress development of atopic dermatitis in NC/Nga mice." *Phytother Res* **26**(4): 594-599.

Lee, I. K., C. J. Kim, K. S. Song, H. M. Kim, H. Koshino, M. Uramoto and I. D. Yoo (1996). "Cytotoxic benzyl dihydroflavonols from Cudrania tricuspidata." *Phytochemistry* **41**(1): 213-216.

Lee, T., S. Lee, K. Ho Kim, K. B. Oh, J. Shin and W. Mar (2013). "Effects of magnolialide isolated from the leaves of Laurus nobilis L. (Lauraceae) on immunoglobulin E-mediated type I hypersensitivity in vitro." *J Ethnopharmacol* **149**(2): 550-556.

Lev, E. and Z. Amar (2000). "Ethnopharmacological survey of traditional drugs sold in Israel at the end of the 20th century." *J Ethnopharmacol* **72**(1-2): 191-205.

Li-Weber, M., M. Giaisi, M. K. Treiber and P. H. Krammer (2002). "The anti-inflammatory sesquiterpene lactone parthenolide suppresses IL-4 gene

expression in peripheral blood T." *Eur J Immunol* **32**(12): 3587-3597.

Loi, M. C., F. Poli, G. Sacchetti, M. B. Selenu and M. Ballero (2004). "Ethnopharmacology of ogliastra (villagrande strisaili, sardinia, Italy)." *Fitoterapia* **75**(3-4): 277-295.

MacGlashan, D. W., Jr., B. S. Bochner, D. C. Adelman, P. M. Jardieu, A. Togias, J. McKenzie-White, S. A. Sterbinsky, R. G. Hamilton and L. M. Lichtenstein (1997). "Down-regulation of Fc(epsilon)RI expression on human basophils during in vivo treatment of atopic patients with anti-IgE antibody." *J Immunol* **158**(3): 1438-1445.

Marco, J. A., J. F. SanzCervera, V. GarciaLliso and N. Batlle (1997). "Sesquiterpene lactones from Artemisia lucentica." *Phytochemistry* **45**(4): 755-763.

Mastuda, H., T. Morikawa, K. Ueda, H. Managi and M. Yoshikawa (2002). "Structural requirements of flavonoids for inhibition of antigen-Induced degranulation, TNF-alpha and IL-4 production from RBL-2H3 cells." *Bioorg Med Chem* **10**(10): 3123-3128.

Matsubara, M., S. Masaki, K. Ohmori, A. Karasawa and K. Hasegawa (2004). "Differential regulation of IL-4 expression and degranulation by anti-allergic olopatadine in rat basophilic leukemia (RBL-2H3) cells." *Biochem Pharmacol* **67**(7): 1315-1326.

Matsubara, M., S. Masaki, K. Ohmori, A. Karasawa and K. Hasegawa (2004). "Differential regulation of IL-4 expression and degranulation by anti-allergic olopatadine in rat basophilic leukemia (RBL-2H3) cells." *Biochem Pharmacol* **67**(7): 1315-1326.

Metcalfe, D. D., D. Baram and Y. A. Mekori (1997). "Mast cells." *Physiol Rev* **77**(4): 1033-1079.

Mitchell, J. C., B. Fritig, B. Singh and G. H. Towers (1970). "Allergic contact dermatitis from Frullania and Compositae. The role of sesquiterpene lactones." *J Invest Dermatol* **54**(3): 233-239.

Oh, P. S. and K. T. Lim (2010). "Modulatory effects of phytoglycoprotein (75 kDa) on allergic inflammatory cytokines in Di(2-ethylhexyl) phthalate

(DEHP)-stimulated RBL-2H3 cells." *J Cell Biochem* **109**(1): 124-131.

Oliver, C., N. Sahara, S. Kitani, A. R. Robbins, L. M. Mertz and R. P. Siraganian (1992). "Binding of monoclonal antibody AA4 to gangliosides on rat basophilic leukemia cells produces changes similar to those seen with Fc epsilon receptor activation." *J Cell Biol* **116**(3): 635-646.

Oliver, J. M., D. L. Burg, B. S. Wilson, J. L. McLaughlin and R. L. Geahlen (1994). "Inhibition of mast cell Fc epsilon R1-mediated signaling and effector function by the Syk-selective inhibitor, piceatannol." *J Biol Chem* **269**(47): 29697-29703.

Onose, J., C. Xie, Y. Q. Ye, K. Sugaya, S. Takahashi, H. Koshino, K. Yasunaga, N. Abe and K. Yoshikawa (2008). "Vialinin A, a novel potent inhibitor of TNF-alpha production from RBL-2H3 cells." *Biol Pharm Bull* **31**(5): 831-833.

Paolini, R., R. Molfetta, L. O. Beitz, J. Zhang, A. M. Scharenberg, M. Piccoli, L. Frati, R. Siraganian and A. Santoni (2002). " Activation of syk tyrosine kinase is required for c-Cbl-mediated ubiquitination of FcεRI and syk RBL cells,: *J Biol Chem* **277**: 36940-36947.

Park, K. H., Y. D. Park, J. M. Han, K. R. Im, B. W. Lee, I. Y. Jeong, T. S. Jeong and W. S. Lee (2006). "Anti-atherosclerotic and anti-inflammatory activities of catecholic xanthones and flavonoids isolated from Cudrania tricuspidata." *Bioorg Med Chem Lett* **16**(21): 5580-5583.

Passante, E. and N. Frankish (2009). "The RBL-2H3 cell line: its provenance and suitability as a model for the mast cell." *Inflamm Res* **58**(11): 737-745.

Pazdrak, K., B. Olszewska-Pazdrak, S. Stafford, R. P. Garofalo and R. Alam (1998). "Lyn, Jak2, and Raf-1 kinases are critical for the antiapoptotic effect of interleukin 5, whereas only Raf-1 kinase is essential for eosinophil activation and degranulation." *J Exp Med* **188**(3): 421-429.

Pecht, I. and A. Corcia (1987). "Stimulus-secretion coupling mechanisms in mast cells." *Biophys Chem* **26**(2-3): 291-301.

Pieroni, A., C. L. Quave, M. L. Villanelli, P. Mangino, G. Sabbatini, L. Santini, T. Boccetti, M. Profili, T. Ciccioli, L. G. Rampa, G. Antonini, C. Girolamini, M. Cecchi and M. Tomasi (2004). "Ethnopharmacognostic survey on the

natural ingredients used in folk cosmetics, cosmeceuticals and remedies for healing skin diseases in the inland Marches, Central-Eastern Italy." *J Ethnopharmacol* **91**(2-3): 331-344.

Plaut, M., J. H. Pierce, C. J. Watson, J. Hanley-Hyde, R. P. Nordan and W. E. Paul (1989). "Mast cell lines produce lymphokines in response to cross-linkage of Fc epsilon RI or to calcium ionophores." *Nature* **339**(6219): 64-67.

Qu, X., S. M. Miah, T. Hatani, M. Okazaki, N. Hori-Tamura, H. Yamamura, H. Hotta and K. Sada (2005). "Selective inhibition of Fcepsilon RI-mediated mast cell activation by a truncated variant of Cbl-b related to the rat model of type 1 diabetes mellitus." *J Biochem* **137**(6): 711-720.

Rodrigues, A. A. S., M. Garcia and J. A. Rabi (1978). "Chemical-Transformations of Abundant Natural-Products .5. Facile Biomimetic Synthesis of Costunolide-1,10-Epoxide, Santamarin and Reynosin." *Phytochemistry* **17**(5): 953-954.

Sahara, N., R. P. Siraganian and C. Oliver (1990). "Morphological changes induced by the calcium ionophore A23187 in rat basophilic leukemia (2H3)

cells." *J Histochem Cytochem* **38**(7): 975-983.

Saitoh, S., R. Arudchandran, T. S. Manetz, W. Zhang, C. L. Sommers, P. E. Love, J. Rivera and L. E. Samelson (2000). "LAT is essential for Fc(epsilon)RI-mediated mast cell activation." *Immunity* **12**(5): 525-535.

Sampson, A. P. (2001). "IL-5 priming of eosinophil function in asthma." *Clin Exp Allergy* **31**(4): 513-517.

Sandomenico, A., S. M. Monti, R. Palumbo and M. Ruvo (2011). "A new FcepsilonRI receptor-mimetic peptide (PepE) that blocks IgE binding to its high affinity receptor and prevents mediator release from RBL 2H3 cells." *J Pept Sci* **17**(9): 604-609.

Sato, S., T. Katagiri, S. Takaki, Y. Kikuchi, Y. Hitoshi, S. Yonehara, S. Tsukada, D. Kitamura, T. Watanabe, O. Witte and K. Takatsu (1994). "IL-5 receptor-mediated tyrosine phosphorylation of SH2/SH3-containing proteins and activation of Bruton's tyrosine and Janus 2 kinases." *J Exp Med* **180**(6): 2101-2111.

Siraganian, R. P., R. O. de Castro, E. A. Barbu and J. Zhang (2010). "Mast cell signaling: the role of protein tyrosine kinase Syk, its activation and screening methods for new pathway participants." *FEBS Lett* **584**(24): 4933-4940.

Suzuki, Y., T. Yoshimaru, K. Yamashita, T. Matsui, M. Yamaki and K. Shimizu (2001). "Exposure of RBL-2H3 mast cells to Ag(+) induces cell degranulation and mediator release." *Biochem Biophys Res Commun* **283**(3): 707-714.

Takaki, S., A. Tominaga, Y. Hitoshi, S. Mita, E. Sonoda, N. Yamaguchi and K. Takatsu (1990). "Molecular cloning and expression of the murine interleukin-5 receptor." *Embo Journal* **9**(13): 4367-4374.

Tamura, S., K. Yoshihira, K. Fujiwara and N. Murakami (2010). "New inhibitors for expression of IgE receptor on human mast cell." *Bioorg Med Chem Lett* **20**(7): 2299-2302.

Tang, J., S. Sawasdikosol, J-H. Chang and S. J. Burakoff (1999). "SLAP, a dimeric adapter protein, plays a functional role in T cell receptor signaling." *Proc Natl Acad Sci* **96**(17): 9775-9780.

Taylor, J. A., J. L. Karas, M. K. Ram, O. M. Green and C. Seidel-Dugan (1995). "Activation of the high-affinity immunoglobulin E receptor Fc epsilon RI in RBL-2H3 cells is inhibited by Syk SH2 domains." *Mol Cell Biol* **15**(8): 4149-4157.

Theoharides, T. C., W. Sieghart, P. Greengard and W. W. Douglas (1980). "Antiallergic drug cromolyn may inhibit histamine secretion by regulating phosphorylation of a mast cell protein." *Science* **207**(4426): 80-82.

Tian, Y. H., H. C. Kim, J. M. Cui and Y. C. Kim (2005). "Hepatoprotective constituents of Cudrania tricuspidata." *Arch Pharm Res* **28**(1): 44-48.

Togawa, M., M. Kiniwa and H. Nagai (2001). "The roles of IL-4, IL-5 and mast cells in the accumulation of eosinophils during allergic cutaneous late phase reaction in mice." *Life Sci* **69**(6): 699-705.

Twentyman, P. R. and M. Luscombe (1987). "A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity." *Br J Cancer* **56**(3): 279-285.

Velling, P., D. Skowasch, S. Pabst, E. Jansen, I. Tuleta and C. Grohe (2011). "Improvement of quality of life in patients with concomitant allergic asthma and atopic dermatitis: one year follow-up of omalizumab therapy." *Eur J Med Res* **16**(9): 407-410.

Vial, D., C. Oliver, M. C. Jamur, M. V. Pastor, E. da Silva Trindade, E. Berenstein, J. Zhang and R. P. Siraganian (2003). "Alterations in granule matrix and cell surface of focal adhesion kinase-deficient mast cells." *J Immunol* **171**(11): 6178-6186.

White, M. (1999). "Mediators of inflammation and the inflammatory process." *J Allergy Clin Immunol* **103**(3 Pt 2): S378-381.

Wong, B. R., E. B. Grossbard, D. G. Payan and E. S. Masuda (2004). "Targeting Syk as a treatment for allergic and autoimmune disorders." *Expert Opin Investig Drugs* **13**(7): 743-762.

Wu, J. B., Y. T. Chun, Y. Ebizuka and U. Sankawa (1991). "Biologically active constituents of Centipeda minima: sesquiterpenes of potential anti-allergy activity." *Chem Pharm Bull (Tokyo)* **39**(12): 3272-3275.

Yamaguchi, Y., Y. Hayashi, Y. Sugama, Y. Miura, T. Kasahara, S. Kitamura, M. Torisu, S. Mita, A. Tominaga and K. Takatsu (1988). "Highly purified murine interleukin 5 (IL-5) stimulates eosinophil function and prolongs in vitro survival. IL-5 as an eosinophil chemotactic factor." *J Exp Med* **167**(5): 1737-1742.

Yamasaki, S. and T. Saito (2004). "Inhibitory adaptors in lymphocytes." *Semin Immunol* **16**: 421-427.

국문초록

꾸지뽕 열매에서 분리한 5,7,3′,4′ −Tetrahydroxy−6,8−Diprenylisoflavone 과 월계수 잎에서 분리한 Magnolialide의 비만세포에서 면역글로블린 E 매개의 신호전달 조절 기전 연구

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알레르기성 질환에 관여하는 세포들의 정의와 병리적 기전에 대한 이해는 많은 발전을 이뤄오며 치료제 개발을 위한 연구가 진행되고 있다. 그러나 현재 알레르기성 질환 치료제와 치료방법들은 대부분 증상 완화에 초점을 두고 있어 재발의 가능성이 크며, 부작용 사례 또한 빈번하게 보고되는 있는 실정이다. 비만세포는 세포질 내에 과립을 가지고 있는 세포로서 알레르기 염증반응에 관여한다. 비만세포는 IgE−FcεRI 반응에 의해 과립내의 화학 매개체와 싸이토카인 등을 분비하면서 알레르기의 초기 반응과 후기반응

을 조절하며, 만성적으로 염증을 지속시키는데에 주요한 역할을 하고 있다.

이에 본 연구에서는 천연물에서 비만세포의 신호전달을 조절하는 물질을 연구해 새로운 치료제의 가능성을 살펴보았다. 꾸지뽕 열매의 메탄올 추출물 (MFC)와 분리된 단일 물질인 5,7,3′,4′-tetrahydroxy-6,8-diprenylisoflavone (THDPI)이 비만세포의 탈과립을 억제하는 것을 확인하였다. MFC는 IgE와 그 수용체인 FcεRI의 결합을 억제하며, FcεRI의 3 가지 subunit중 β-subunit의 유전자 발현을 감소시켰다. THDPI는 IgE-FcεRI 매개의 신호전달 중 Spleen tyrosine kinase 의 활성을 억제해 LAT/PLCγ의 하위신호를 억제하고, 세포 내 칼슘농도 증가와 F-actin 재배열을 억제하는 것을 확인하였다. 이러한 THDPI의 활성은 Src-like adaptor protein (SLAP)의 발현 증가 및 c-Cbl E3 ligase의 활성을 높여 유비퀴틴-프로테아좀 매개의 단백질 분해작용을 촉진하는 것과 관련이 있는 것을 확인하였다. 월계수 잎에서 분리된 magnolialide는 비만세포의 탈과립 억제와 함께, 아토피 질환의 지표물질 중 하나인 IL-4 의 발현과 분비를 억제하는

것을 확인하였다. 또한 IgE를 생산 하는 B cell의 proliferation을 억제하는 것을 확인하였다.

본 연구를 통해 비만세포의 활성을 조절할 수 있는 천연물로써 꾸지뽕 열매에서 분리된 THDPI와 월계수 잎에서 분리된 magnolialide와 제시하였으며, Syk kinase의 활성 조절을 통한 비만세포 매개의 알레르기성 질환 치료제 개발을 위한 가능성을 제시하였다.

주요어 : 5,7,3′,4′-Tetrahydroxy-6,8-diprenylisoflavone, Immunoglobulin E, FcεRI, Mast cell, magnolialide, SLAP

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